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CONTENTS OF VOLUME 48

No. 1, July, 1960

NOTES

LETTERS TO THE EDITORS

No. 2, August, 1960

LETTERS TO THE EDITORS

No. 3, September, 1960

No. 4, October, 1960

No. 6, December, 1960

LETTERS TO THE EDITORS

The Journal of Biochemistry, Vol. 48, No. 1, 1960

ISOMETRIC AND ISOTONIC STUDIES ON CONTRACTION AND RERAXATION OF GLYCEROL-TREATED MUSCLE FIBER OF RABBIT PSOAS*

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(Received for publication, August 17, 1959)

It is observed that the glycerol-treated muscle fiber of rabbit *psoas* shortened in a bath of isotonic concentrations of KCl containing adenosinetriphosphate (ATP)***** and magnesium ions (Mg^{++}) shows almost no relaxation under isotonic conditions (loaded 0.4~0.5 kg/cm^2) even after removing ATP and Mg^{++} from the bath but that the fiber contracted by ATP and Mg^{++} under isometric conditions is made to relax to a considerable extent by mere washing with isotonic concentrations of KCl containing neither ATP nor Mg^{++}. In this connection, some of these observations that the glycerol-treated fibers respond to an application of some reagents differently under isotonic and isometric conditions, are going to be reported in this paper.

MATERIALS AND METHODS

The glycerol-treated muscle fiber was prepared from rabbit *psoas* in the same way as described in the previous papers (*1*, *2*). The fiber was washed, before it was hung on a lever, with 0.15 *M* KCl buffered with 0.05 *M* 2,4,6-trimethylpyridine (TMP) (pH 7.1) at 0°. The diameter of the fiber bundles used for each run was about 200 μ.

In the isotonic study, a kymography with smoked drum was used and in the isometric study, a mechano-electronic transducer tube RCA 5734, and a NIHONKŌDEN D.C. amplifier and recorder were employed.

The former study was carried out at room temperature (20~27°) without special control of temperature, while the latter was done in a water bath of constant temperature at 25°. In both cases, the fiber bath contains always 0.15 *M* KCl and 0.05 *M* TMP buffer and sometimes various concentrations of ATP, Mg^{++}, ethylenediaminetetraacetic acid (EDTA), and others, to a final volume of 5 ml. (in a few cases 3 ml.). The medium was stirred by bubbling nitrogen gas through it. The contraction and relaxation

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***** The following abbreviations are used in this paper: ATP for adenosinetriphosphate, TMP for 2,4,6-trimethylpyridine and EDTA for ethylenediaminetetraacetate.

were expressed in the percentages of initial length of the fiber used in the isotonic experiments and in grams per cm^2 of the cross-section area of the fiber in the isometric experiments.

RESULTS

Effect of Washing on the Contracted Fiber—The fiber in the bath of ATP alone shows a small contraction and contracts very nicely upon subsequent addition of magnesium ions. Here, Mn^{++}, Co^{++} or Ni^{++} can substitute for Mg^{++}, while Ca^{++}, Sr^{++}, Zn^{++} or Cd^{++} cannot (see *2*). These ion effects are observed under either isotonic or isometric conditions. However, when the fiber bath containing ATP and divalent cations is replaced by a bath without them, the tension developed under isometric conditions decreases down to about 50 per cent (variably from fiber to fiber) of the maximum tension developed, while the fiber shortened under isotonic conditions maintains its shortended length.

As an example, the isometric contraction by ATP and Mg^{++} which is very quick compared with the isotonic contraction and the relaxation by mere washing are shown in Fig. 1. The relaxation is very slow but the

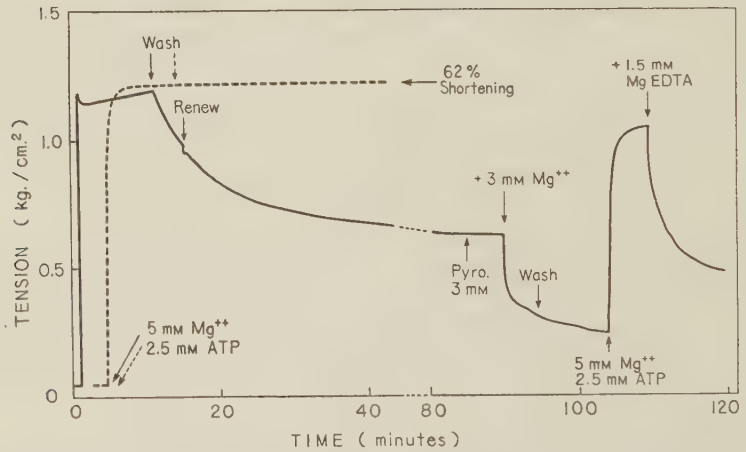

FIG. 1. Effect of washing on the contracted fiber. The relaxation is observed by mere washing under isometric conditions (full line), while not under isotonic conditions (dotted line). A relatively low concentration of pyrophosphate with Mg^{++} produces a rapid relaxation under isometric conditions.

second contraction indicates that this is not a destructive process but a fairly reversible relaxation. Therefore, this seems to be a significant observation. It should be mentioned here, however, that this decrease in tension is not a new observation. For example, Weber *et al.* (*3*), stated that if the ATP was washed out of the models when contracted, 70~80 per cent of the tension and the whole of the shortenig remained. In other words, 20~30 per cent of the tension is lost by washing, while there is no loss in the shortening.

Characteristic Effect of Cd^{++} and Zn^{++} Ions—It has been reported (*2*, *4*) that some divalent cations such as Mn^{++}, Ca^{++}, Cd^{++}, Zn^{++} but not Mg^{++} have the effect of causing a contraction of the fiber relaxed in a medium of EDTA, ATP and Mg^{++} upon their addition to it. Three more cations, Ni^{++}, Co^{++} and Sr^{++} have now been tried and the specificity of Ca^{++} and Sr^{++} ions in reversing the EDTA relaxation has been emphasized further: the fiber relaxed in the medium containing EDTA starts to contract upon addition of Ca^{++} in a concentration much less than that of the EDTA present, while for Mn^{++}, Co^{++}, Ni^{++}, Cd^{++} and Zn^{++} ions, their concentration must be increased to nearly that of the EDTA present to reverse the EDTA relaxation.

Also, Cd^{++} and Zn^{++} ions were found to be distinguishable from the other divalent cations in that the fiber relaxes again spontaneously when the concentration of Cd^{++} or Zn^{++} exceeds that of EDTA present. This second relaxation by Cd^{++} or Zn^{++} can be reversed by the addition of cysteine but not by that of histidine (Fig. 2). In contrast with these observations on

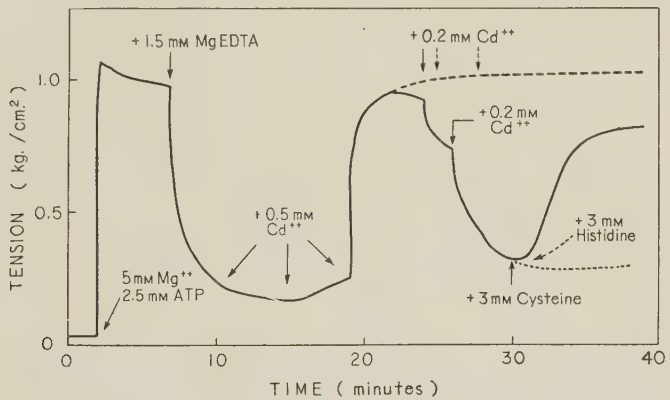

Fig. 2. Effect of Cd^{++} ions on the EDTA relaxation. The reversal of the EDTA relaxation starts when the concentration of Cd^{++} reaches nearly that of the EDTA present. Subsequent addition of Cd^{++} causes a spontaneous relaxation only under isometric conditions (dotted line for isotonic contraction) which can be reversed by the addition of cysteine while not by that of histidine.

isometric contractions, when the fiber is allowed to shorten under isotonic conditions, the spontaneous relaxation by Cd^{++} or Zn^{++} is not observed even when the concentration of Cd^{++} or Zn^{++} exceeds that of the EDTA present (see the dotted curve in Fig. 2)

Lengthening by Cysteine The following observation is not necessarily related to the difference between the isotonic and the isometric contraction since it has been studied only under isotonic conditions. When a fiber shortened and washed was treated with a rather high concentration of cysteine, lengthening of the fiber was observed, and it was enhanced by addition of Ca^{++} or Mg^{++}. This lengthening could be halted by monoiodoacetate but not by

mere washing (*5*). Further observations are now made to emphasize the specificity of cysteine (SH compound) in this lengthening: neither ascorbic acid nor histidine can substitute for cysteine. Zn^{++} or Cd^{++} can also halt the lengthening as did monoiodoacetate.

A fiber once lenghened in a medium containing cysteine loses its recontractility partially or completely and this was the reason why one of the authors employed the term "lengthening" instead of relaxation (*5*). However, it is now observed that a fiber lengthened in the medium of cysteine and Ca^{++} can contract again perfectly as shown in Fig. 3. Here, the fiber is

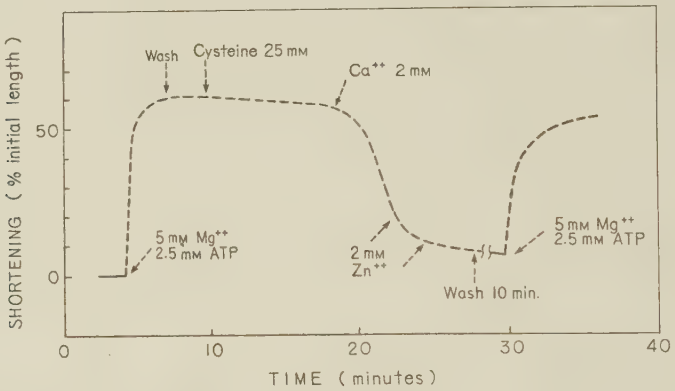

FIG. 3. Recontractility of the fiber lengthened by cysteine and Ca^{++}. The lengthening is stopped by the addition of Zn^{++} ions, and the fiber shows then a perfect contraction in the medium of ATP and Mg^{++}.

lengthened in a medium of cysteine and Ca^{++}. The lengthening is halted by Zn^{++} (or Cd^{++}) before the fiber is extended back to its initial length. The fiber is then washed for 10 minutes and the standard contraction medium of ATP plus Mg^{++} is applied to the fiber.

It is important to point out what procedure among those mentioned above is the key point to make the lengthening reversible. In Table I, is shown a series of experiments which is intended to find the conditions for the cysteine lengthening and for the recontractility of the lengthened fibers. The results are not always consistent with each other and even the cysteine lengthening is not always observed. However, it seems at present that at least two conditions are necessary to obtain a good reversibility of the cysteine lengthening: the fiber should be lengthened rather fast within 30 minutes and the lengthening must be stopped by Cd^{++} or Zn^{++} instead of monoiodoacetate.

Relaxing Effect of Inorganic Pyrophosphate—A report was also made of the relaxing effect of inorganic pyrophosphate under isotonic conditions (*5*). There, a very high concentration of pyrophosphate ($20 \sim 30 \times 10^{-3}$ *M*) was needed to produce a relaxation in the presence of Mg^{++}, and the relaxation was rather sluggish.

Under isometric conditions, in contrast with isotonic conditions, a rapid relaxation is observed with a low concentration of pyrophosphate (3×10^{-3} *M*) as seen in Fig. 1 (between the first and the second cycle of contraction).

Necessity of Mg^{++} Ions for the Contraction by Cd^{++} or Zn^{++}—As in the contraction with Ca^{++}, the presence of Mg^{++} is necessary for Cd^{++} or Zn^{++} to produce a contraction of the fiber relaxed in the medium of EDTA. The evidence for this necessity of Mg^{++} given in the experiment shown in Fig. 4 is not direct but is an interesting one. ATP and Cd^{++} (or Zn^{++}) are now

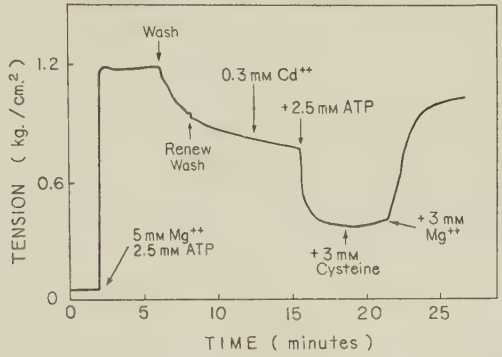

FIG. 4. Necessity of Mg^{++} ions for the contraction in the presence of Cd^{++} ions.

ATP and Cd^{++} produce a relaxation which is not reversed by the addition of cysteine alone unless Mg^{++} ions are also added.

employed in place of ATP, Mg^{++} and EDTA to produce a relaxation of the fiber contracted by ATP and Mg^{++} and washed for 10 minutes. The addition of cysteine does not, however, reverse the relaxation unless Mg^{++} ions are also added. It may be noticed that the fourth addition of cysteine in Fig. 4 causes a small contraction but it is likely that this small contraction is the same kind of contraction as is usually observed in the medium of ATP alone. It should also be mentioned here that the concentration of Cd^{++} or Zn^{++} must be lower than that of ATP for being able to obtain the relaxation by ATP and Cd^{++} or Zn^{++}.

In spite of the previous observation of the Cd^{++} effect shown in Fig. 2, this relaxation by Cd^{++} plus ATP can also be observed under isotonic conditions although there are some experiments suggesting that the higher the magnitude of shortening of the fiber by ATP plus Mg^{++}, the smaller the magnitude of relaxation by ATP plus Cd^{++} under isotonic conditions.

DISCUSSION

The first three observations are that the isotonic shortening can be reversed by cysteine (Fig. 3) but not by mere washing (Fig. 1) nor by concentrations of Cd^{++} over that of the EDTA present (Fig. 2). These observa-

tions seem to suggest that in the isotonic shortening a secondary reaction takes place in addition to the reactions that are supposed to be responsible for the tension development in the muscle fiber. This secondary reaction

TABLE I

Cysteine Lengthening and Recontractility

The results obtained with 19 samples of fiber bundles are shown. The fibers have been shortened once in the medium of ATP and Mg^{++} and have been lengthened by a rather high concentration of cysteine in the presence of Mg^{++} or Ca^{++} as an accelerator for the lengthening. (a) Two of these fibers (No. 1 and 2) do retain their full recontractility after the cysteine lengthening has been stopped by Zn^{++}. (b) Two of the fibers (No. 15 and 16) that have been shortened in the first place by less than 40 per cent of the initial length of fibers do not show any lengthening by cysteine, while all of the fibers that have been shortened by more than 40 per cent (No. 3~13) do show the lengthening to some extent. (The case with the fiber No. 14 is neglected on the assumption that if it were treated with cysteine for more than 11 minutes, it would have been lenghthened to some extent.) (c) Among the fibers that have been lengthened rapidly, those whose cysteine-lengthening have been stopped by Zn^{++} (or Cd^{++}) show a better contractility than those without the Zn^{++} (or Cd^{++})-treatment.

No.	Diameter of Fibers (mm)	Washing before Contraction Run (hours)	Isotonic Contraction (% of initial length)	Duration of Cysteine Application (min.)	Cations added		(% of initial length)	
					For Lengthening	For Interruption of Lengthening	Shortening Remained when Lengthening is Stopped	Recontraction
1	0.15	0.2	34	28	Ca^{++}	Zn^{++}	6.5	60
2	0.20	4.0	38	15.5	Mg^{++}	Zn^{++}	0	44
3	0.18	8.7	53	15	Ca^{++}	Zn^{++}	0	39
4	0.20	6.0	63.5	60	Ca^{++}	Zn^{++}	8.4	37.5
5	0.20	4.5	65	18.5	Mg^{++}	none	3.5	10,5
6	0.25	6.5	62.5	86	Ca^{++}	none	15.0	27.5
7	0.20	6.0	55	44	Mg^{++}	none	26.5	40
8	0.20	1.0	57.5	100	Mg^{++}	none	25	40.5
9	0.20	7.8	37	21.5	Mg^{++}	none	28	—
10	0.20	5.5	48	76	Ca^{++}	none	40	—
11	0.18	4.3	60	23	Ca^{++}	none	54	—
12	0.18	4.0	53	36	Ca^{++}	none	39	—
13	0.25	4.3	57	64	none	none	41	—
14	0.20	9.0	63	11	Mg^{++}	none	63	—
15	0.20	8.3	34	21	Mg^{++}	none	34	—
16	0.20	0.5	38	43.5	Ca^{++}	none	38	—

might be, for example, the formation of S-S linkage (or NH-Mg-S, S-Mg-S). This kind of cross-linkage would hardly be split by mere washing or by Cd^{++} and therefore, the fiber would not be lengthened. On the other hand, it might be opened up by cysteine.

The fourth observation that the relaxation by inorganic pyrophosphate is sluggish under isotonic conditions while it is rapid under isometric conditions could be taken as another support for the formation of a cross-linkage.

However, Briggs (see *6*), using a single fiber preparation, has recently shown that inorganic pyrophosphate and Mg^{++} can produce a relaxation only when the natural microsomal relaxing factor is also present. Therefore, the fourth observation mentioned above may be understood from the two points of view: one, the preparation of glycerol-treated fiber bundles used here still contain the so-called natural relaxing factor studied especially by Ebashi (*7*), Portzehl, Briggs (*8*) and others. Two, the diameter of fiber bundles is kept almost constant under isometric conditions but it becomes much larger in the isotonic shortening. These two points of view can explain not only the observation on the pyrophosphate relaxation but also at least partially the preceding observations shown in Figs. 1 and 2; that is, the effect of washing and that of Cd^{++}. Furthermore, the isometric relaxation by Cd^{++} plus ATP shown in Fig. 4 and the results shown in Table I make the earlier suggestion of the formation of a cross-linkage uncertain. As to the cysteine lengthening which is the most important observation for inspiring the idea of the cross-linkage formation, there are at least two additional points unsettled. One is, as mentioned in the previous paper (*5*), that the recontractility of the lengthened fiber (see Fig. 3) might be due to the core of the fiber bundle that was unstimulated in the first cycle of contraction. The other is that cysteine would fould form S-S linkage with the SH groups of myosin rather than open up the S-S linkage of the protein (*cf.* *9*).

Therefore, to summarize, there are differences in observations between isotonic and isometric contractions but it is obscure whether these are substantial differences between isotonic and isometric conditions, or whether they are just artifacts caused by the extreme shortening which is possible with glycerol-treated fibers.

SUMMARY

1. It is observed that the isometric contraction of the glycerol-treated fiber in a bath containing ATP and Mg^{++} is reversed to a considerable extent by mere washing, while the fiber contracted under isotonic conditions remains shortened upon washing.

2. A previous report (*4*) has stated that the fiber relaxed in a bath of EDTA, ATP and Mg^{++} can be made to contract upon addition of Cd^{++} or Zn^{++}, but that the fiber relaxes again spontaneously when the concentration of Cd^{++} or Zn^{++} added exceeds that of the EDTA present. In contrast with these observations under isometric conditions, the second relaxation by Cd^{++} or Zn^{++} is not observed.

3. The pyrophosphate relaxation of the fiber under isometric conditions requires a concentration of pyrophosphate much lower and proceeds faster than that under isotonic conditions.

4. The cysteine-lengthening of the fiber under isotonic conditions can be stopped by addition of Cd^{++} or Zn^{++}. The fiber thus ceased its lengthening retains the full recontractility.

5. A discussion is made on a temporary suggestion of the cross-linkage formation in the fiber under isotonic conditions.

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BIOCHEMICAL STUDIES ON THE FORMATION OF THE SILKPROTEIN

VIII. THE SYNTHESIS OF α-KETOGLUTARIC ACID, OXALACETIC ACID, AND GLYOXYLIC ACID FROM GLUCOSE IN THE SILKWORM

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(Received for publication, October 8, 1959)

In mammals, it has been elucidated that α-ketoglutaric acid, oxalacetic acid, and pyruvic acid are physiologically important intermediates which link between carbohydrate metabolism and protein metabolism.

In silkworms, it has been elucidated that glyoxylic acid (*1*, *2*), which is not usually found in mammals, α-ketoglutaric acid (*1*), and oxalacetic acid (*1*) exist in the body fluid, but the existence of pyruvic acid (*1*) in silkworms is not yet proved. Furthermore, it has been pointed out that these keto acids, including pyruvic acid, take part in the synthesis of amino acids in the silkworm body, and that these synthesized amino acids are also utilized for the synthesis of silkprotein (*3-10*). Little is known, however, of precursors for the synthesis of the keto acids in the silkworm body.

The current work was carried out, using C^{14} glucose, to examine whether α-ketoglutaric acid, oxalacetic acid, and glyoxylic acid are synthesized from glucose in the silkworm. Three kinds of keto acid were respectively isolated from the body fluid of the silkworms which consumed C^{14} glucose; their radioactivities were then measured.

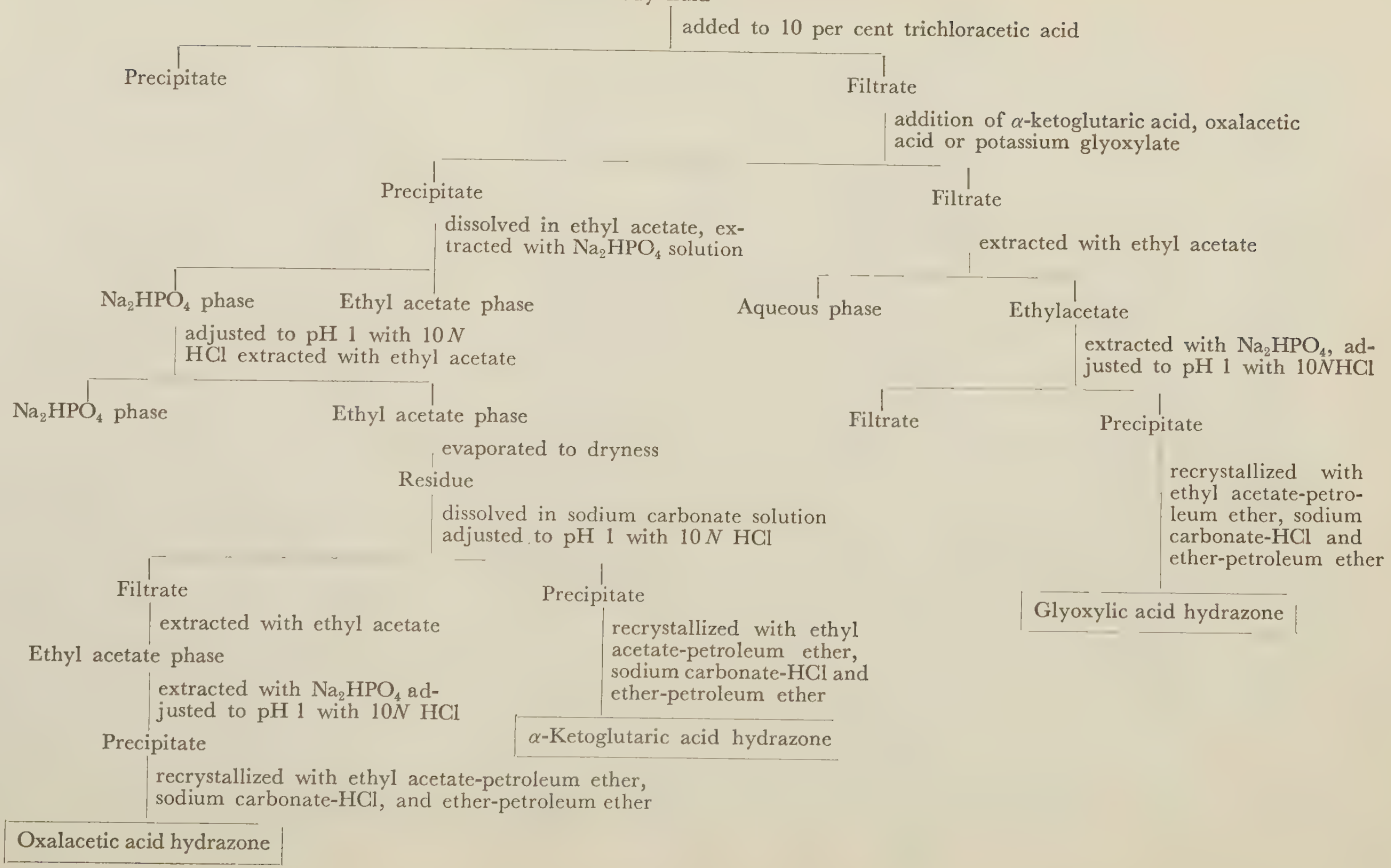
EXPERIMENTAL

Isolation of keto acids from the body fluid: glucose all labeled with C^{14} (12mc./mM) was obtained from the Radiochemical Centre, Amersham, England. A half μc. of C^{14} glucose per worm was given per os to the silkworm at the 3rd day of the 5th instar (N122×C115). Silkworms were reared on mulberry leaves for five hours, and then 30 ml. of the body fluid of silkworms was added to 270 ml. of 10 per cent of trichloracetic acid, and its proteins were precipitated. The supernatant solution was divided into three equal parts, and to each part were respectively added 50 mg. of α-ketoglutaric acid, 50 mg. of oxalacetic acid or 100 mg. of potassium glyoxylate for carrier, and then 15 ml. of 2*N* hydrochloric acid solution of 2,4-dinitrophenylhydrazine were added. After standing three weeks in a refrigerator, each keto acid hydrazone was isolated according to the procedure shown in Table 1, and each hydrazone was recrystallized until its specific activity became constant. The radioactivity of the isolated hydrazones was measured with SC-16 Windowless Gas Flow Connter (Tracerlab. Inc.) and its identification was done by chromatography.

* Tokyo Metropolitan University

TABLE I

Isolation of Keto Acids from the Body Fluid of the Silkworm



RESULTS AND DISCUSSION

Three kinds of hydrazone isolated from the body fluid of silkworms which consumed C^{14} glucose were analyzed by paper chromatography with *n*-butanol solution saturated with water as a solvent. Their R_f values were 0.08, 0.13 and 0.24 which agreed with those of authentic specimens of α-ketoglutaric acid hydrazone, oxalacetic acid hydrazone and glyoxylic acid hydrazone (Table II).

TABLE II

Paper Chromatogram of Keto Acid Hydrazone

Hydrazone	R_f value	
	Experimental	Authentic Specimen
α-Ketoglutaric acid	0.08	0.08
Oxalacetic acid	0.13	0.12
Glyoxylic acid	0.24	0.24

The yield, and the radioactivity of each hydrazone of α-ketoglutaric acid, oxalacetic acid, and glyoxylic acid isolated from the body fluid of the silkworm are shown in Table III. Extremely high radioactivity was recognized in α-ketoglutaric acid and then in oxalacetic acid. Recently, as the existence of the glycolysis (*11*) and the citric acid cycle (*12*) has been pointed out at the midgut-cell of the silkworm, the formation of α-ketoglutaric acid and oxalacetic acid from glucose seems to take place by passing through these two metabolic pathways.

TABLE III

Radioactivity of the Keto Acid Isolated from the Body Fluid

Keto Acid	Yield (as hydrazone)	Radioactivity	
		c.p.m. mg. of hydrazone[1)]	c.p.m. mg. of keto acid[2)]
	mg.		
α-Ketoglutaric acid	44.8	505	95108
Oxalacetic acid	1.9	38	44840
Glyoxylic acid	5.1	2.7	162

1) c.p.m. per mg. of the isolated hydrazone
2) c.p.m. per mg. of keto acid present in the body fluid

Radioactivity of glyoxylic acid was very low, as compared with that of α-ketoglutaric acid and oxalacetic acid. This facts seems to suggest that, though the carbon of glucose is, in fact, used for formation of the carbon of glyoxylic acid in the silkworm, the activity of this reaction is very weak.

As it has been pointed out that glyoxylic acid, which is not usually found in the blood of mammals, exists in comparatively high content in the body fluid of the silkworm (*2*), and that glyoxylic acid converts to glycine in the silkworm body (*2*, *10*), the finding of precursors of glyoxylic acid seem to be of importance in relation to the biosynthesis of glycine (*1*, *10*, *13*, *14*), one of the main constituent amino acids of the silkprotein.

Though it is pointed out in the current study that glucose is one of the precursors of glyoxylic acid synthesis in the silkworm body, it seems to be doubtful that this conversion is the main pathway for glyoxylic acid synthesis. Recently the authors isolated glycine from the silkprotein produced by silkworms which consumed 0.5 μc. of C^{14} glucose at the 3rd day of the 5th instar. This isolated glycine had, in fact, radioactivity in its molecule, but its radioactivity was not so high (56 c.p.m. per mg.).

SUMMARY

1. The current work was carried out, using C^{14} glucose, to examine whether α-ketoglutaric acid, oxalacetic acid, and glyoxylic acid are synthesized from the glucose in the silkworm. Three kinds of keto acid were respectively isolated from the body fluid of silkworms which consumed C^{14} glucose as their 2,4-dinitrophenylhydrazones, and their radioactivities were measured.

2. High radioactivity was recognized in α-ketoglutaric acid and in oxalacetic acid, but the radioactivity of glyoxylic acid was extremely low, showing that glucose is not a direct precursor for glyoxylic acid.

3. It seems to be doubtful that the formation of glyoxylic acid from glucose is the main pathway for glyoxylic acid synthesis.

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STUDIES ON THE METHYLATION OF PYRIDINE-COMPOUND IN ANIMAL-ORGANISMS

V. COMPARISON WITH THE BEHAVIOUR OF NICOTINIC ACID IN SOME VERTEBRATE ANIMALS*,**

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(Received for publication, November 9, 1959)

It is well known that the biological N-methylation of the pyridine ring, for example, N-methylpyridine from pyridine, is a general reaction in the Vertebrate animals, *viz.* Mammalia, Aves, Reptilia and Amphibia (*1-9*). This may be a process to detoxify the bases such as pyridine, quinoline and some other related compounds. In the case of nicotinic acid it is unknown whether this process takes place in all Vertebrate animals or not.

When nicotinic acid was administered to calf, dog, rabbit, lamb, rat, guinea-pig, rhesus monkey and human, it was partly converted to nicotinamide, which was further methylated into N^1-methylnicotinamide, while a part of nicotinic acid was converted to nicotinuric acid, the conjugate of glycine, in dog, rabbit, rat and guinea-pig (*10-14*). According to a report in the old time the metabolism of nicotinic acid in the fowl seemed to be different from these, because the fowl did not excrete nicotinuric acid (*15*). This led us to study on the N-methylation in rabbit, fowl, tortoise and frog bodies for finding whether there is difference between classes or species or does not.

In the present paper it has been attempted to make a comparative study of the behaviour of nicotinic acid in rabbit, fowl, tortoise and frog by using a paper chromatographic technique.

EXPERIMENTALS AND RESULTS

I. Preparation of Samples The following animals were used in this study: rabbit (*Lepus cuniculus* in Mammalia), fowl (*Gallus domesticus* in Aves), tortoise (*Clemmys japonica* in Reptilia), and frog (*Rana nigromaculata* and *R. catesbiana* in Amphibia).

A 24 hours urine was collected from each animal after administration of 0.5 *g.* of nicotinic acid prepared as 10 per cent of its sodium salt solution. After addition of ethanol to the urine, it was filtered through a piece of silk

* A part of this work was read at the annual meeting of the Zoological Society of Japan, held at Tokyo in October, 1959.

** Contributions from the Labolatory of Biological Institute, Kobe University, Kobe, No. 75.

cloth to remove the coarse materials. The filtrate was centrifuged and the separated organic phase was concentrated *in vacuo* to a syrup. This was again filtered and dried. The residue was extracted with fresh alcohol or acetone and the filtrate of this extract was used for paper chromatographic samples.

II. Paper Chromatography The separation of the metabolites of nicotinic acid in the samples is made by paper chromatography. Ten to forty microliters of each sample was spotted on a sheet of Toyo filter paper No. 51 (40×40 *cm.*) and the following solvents were used for developing.

1: *iso*-Butanol: 98 per cent Formic acid: Water = 100 : 20 : 45 in vol.
2: *n*-Butanol: Acetone: Water = 45 : 5 : 50 in vol.
3: 80 per cent Acetone

The chromatograms were obtained by the one dimensional ascending technique and dried after running for 13 hours at 20-25°. Then they were treated with PABA-CNBr according to the methods of previously described in this series (*16*). A resultant chromatogram is shown in Fig. 1.

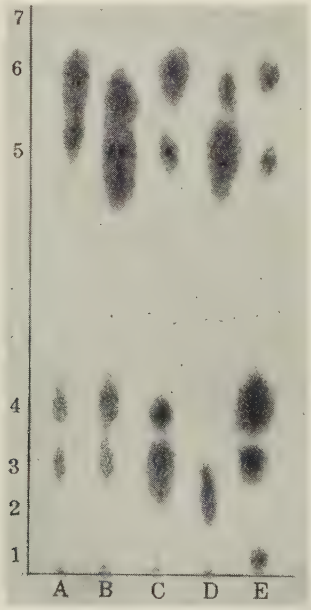

Fig. 1. A chromatogram of urinary excretion from rabbit, fowl, tortoise and frog treated with nicotinic acid. A, Frog (*R. nigromaculata*); B, Bullfrog (*R. catesbiana*); C, Tortoise (*Clemmys japonica*); D, Fowl (*Gallus domesticus*); E, Rabbit (*Lepus cuniculus*).

The following 6 different substances were found, namely, nicotinamide, N^1-methylnicotinamide, trigonelline, N^1-methyl-6-pyridone-3-carboxamide, nicotinuric acid and nicotinoyl-ornithine-like substance. They are identified by their characteristic positions and colors on the chromatograms, comparing

them with those of authentic samples and also with the R_f values published by Thomas and others (*17–21*).

The R_f values of these metabolites in the above-mentioned solvent system are shown in Table I.

TABLE I

The R_f Values of Resultant Spots in Various Solvent Systems

Solvnet system	1	2	3
Nicotinamide	0.55	0.67	0.78
N^1-methyl-nicotinamide	0.12	0.05	0.47
Trigonelline	0.16	0.07	0.16
Nicotinuric acid	0.18	0.13	0.44
Nicotinoyl-ornithine	0.14	—	—
N^1-methyl-6-pyridone-3-carboxamide	0.79	—	—

The metabolic patterns in rabbit, fowl, tortoise and frog are summarized as shown in Table II.

TABLE II

Essential Metabolites in Rabbit, Fowl, Tortoise and Frog Given Nicotinic Acid

Animal	Rabbit	Fowl	Tortoise	Frog	Position in Fig. 1
Nicotinic acid	+	+	+	+	6
Nicotinamide	+	+	+	+	5
N^1-methyl-nicotinamide	+	−	+	+	3
Trigonelline	+	−	−	−	1
Nicotinuric acid	+	−	+	+	4
Nicotinoyl-ornithine-like substance	−	+	−	−	2
N^1-methyl-6-pyridone-3-carboxamide	+	−	−	−	7

DISCUSSION

As described in the previous report, this method is suitable for the preparations of the samples to be employed for detection of the metabolic products of nicotinic acids, furthermore, the procedure of which is simple and the loss of the substances in the urine is minimum. Of course, in this case, the contained admixture in the samples did not disturb the detection of the metabolites. And for paper chromatographical detection of nicotinic acid derivatives, PABA-CNBr treatment gave a satisfactory result, but N^1-methyl-6-pyridone-3-carboxamide and trigonelline were not detected by this method under usual light. Therefore, as for the detection of spots of these

substances, it was only able to ascertain them under the ultraviolet light with appropriate filter.

Upon administration of nicotinic acid to the rabbit, tortoise and frog, nicotinuric acid is excreted in the form of conjugates with glycine, although this does not in the case of the fowl. Author assumes glycine may play an important part in the detoxication of nicotinic acid. Baldwin *et al.* reported that birds excrete dinicotinoylornithine when fed nicotinic acid, analogous to ornithuric acid excretion after benzoic acid administration (*22*). In the present study, there is a new spot regularly appears a little lower than that of nicotinuric acid in the fowl. The present writer has not yet obtained an authentic sample of nicotinoylornithine, but probably it is an ornithine-compound, although he can not say for certain.

Results summarized are as follow:

1. The methyl group in nicotinamide is linked to the nitrogen atom of pyridine ring.
2. N^1-methylnicotinamide is detected in the urines of rabbit, tortoise and frog, while its 6-pyridone derivative is found in the rabbit urine only.
3. It is probably presumable that nicotinamide is converted to N^1-methylnicotinamide by the biological methylation in animal body and one of it's oxidized metabolites is N^1-methyl-6-pyridone-3-carboxamide.

On the other hand, N^1-methylnicotinamide of which the methyl-group is linked to the nitrogen atom in pyridine ring of nicotinamide contained in the urine of the rabbit, tortoise and frog, and its 6-pyridone derivatives being found only in the rabbit urine. The N^1-methylnicotinamide is resultant product of methylation of nicotinamide, and N^1-methyl-6-pyridone-3-carboxamide is its oxidized substance.

SUMMARY

1. An attempt has been made to compare the behaviours of nicotinic acid in rabbit, domestic fowl, tortoise and frog by the chromatographic techniques.

2. The rabbit, fowl, tortoise, and frog converts nicotinic acid into nicotinamide, which is partly methylated into N^1-methylnicotinamide in rabbit, tortoise, and frog except fowl showing quantitative difference and other part of it is oxidized to N^1-methyl-6-pyridone-3-carboxamide in rabbit.

3. In fowl, nicotinic acid seems to be conjugated with ornithine into nicotinoyl-ornithine-like substance, while in rabbit, tortoise and frog it is conjugated with glycine.

The author wishes to express his cordial thanks to Dr. M. Tomita for his continuous advice and encouragement.

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ENZYMATIC STUDIES ON PYRIDOXINE METABOLISM

II. A PYRIDOXINE OXIDIZING ENZYME SYSTEM

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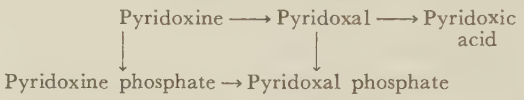
(Received for publication, November 28, 1959)

On the basis of a number of nutritional experiments on microorganisms (*1*) and animals (*2*), two pathways of pyridoxal phosphate formation from pyridoxine have been assumed to be possible as shown in the scheme.



Previous investigation (*3*) revealed the presence of two distinct enzymes in rabbit liver extracts. The one oxidized pyridoxine to pyridoxic acid in the presence of aldehyde oxidase and the other pyridoxine phosphate to pyridoxal phosphate. From a comparison of activities of these two enzymes, it was suggested that ingested pyridoxine would be converted to pyridoxal phosphate via pyridoxine phosphate.

The present paper deals with the purification, properties and some aspects of the reaction mechanism of the enzyme system which oxidizes pyridoxine.

EXPERIMENTAL

Materials and Methods—Pyridoxine hydrochloride was kindly given by Dr. Matsukawa of Takeda Pharmaceutical Company, Osaka.

Pyridoxal was determined fluorometrically as 4-pyridoxic acid, formed from pyridoxal by aldehyde oxidase, which was prepared from rabbit liver according to a modification of Hurwitz's method (*4*). 4-Pyridoxic acid was determined in a Photovolt fluorometer model No. 54 (filters; HG-1 and N 440). A solution of 0.27 μg. of quinine sulfate per ml. of 0.1 *N* H_2SO_4 was employed as a reference standard. Occasionally the pyridoxal concentration was checked with Snell's method using ethanolamine (*5*).

*Purification of Fraction PO**—150 g. of liver from desanguinated rabbits were ground with quartz sand, and extracted with 300 ml. of cold distilled water. The turbid red brown supernatant (356 ml.), obtained after centrifugation at 13,000$\times g$ for 20 minutes, was adjusted to pH 5.0 with 1 *M* acetic acid. The bulky precipitate formed was centrifuged off. The supernatant solution (320 ml.) was adjusted to pH 7.0 with 1 *N* NaOH

* The protein fraction which is necessary for the conversion of pyridoxine to pyridoxic acid in the presence of aldehyde oxidase preparation will be tentatively designated as Fraction PO (Fraction necessary for pyridoxine oxidation).

and 119 ml. of 99 per cent ethanol was added gradually under vigorous stirring at −5°. The precipitate was collected by a brief centrifugation and dissolved in cold 0.05 *M* phosphate buffer, pH 6.0, and dialyzed against buffer of the same composition overnight in the cold. The insoluble part was centrifuged off. To the dialyzed solution, calcium phosphate gel was added at a gel/protein ratio of 1.0. The gel was removed by centrifugation. Usually, the supernatant retained nearly all the activity involved in the oxidation of pyridoxine to pyridoxic acid in the presence of an aldehyde oxidase preparation, but sometimes the activity was markedly reduced. Data of a typical purification procedure are shown in Table I.

TABLE I

Purification of Fraction PO

Procedure	Volume	Protein	Specific activity[1)]	Yield
	ml.	mg.		%
Crude extract	356	16376	0.004	100
Acid treated supernatant	320	5888	0.009	85
0–30 % Ethanol fraction	42	1260	0.035	62
Calcium phosphate gel-treated	40	86	0.21	42

1) μM of pyridoxic acid formed per mg. of protein in ten minutes.

Assay mixture contained, in 2.0 ml., 20 μM of pyridoxine, 1.3 mg. of aldehyde oxidase from rabbit liver, 0.2 ml. of enzyme preparation and 100 μM of phosphate buffer, pH 6.0. Incubation was carried out for 30 minutes at 37°.

Further concentration of Fraction PO activity has not been achieved because no activity was found in any fraction obtained by treatment with ammonium sulfate, and neither alumina C_γ gel nor calcium phosphate gel was able to adsorb Fraction PO. In the case of ammonium sulfate fractionation, neither combinations of all the fractions which were dialyzed nor addition of dissociable cofactor (*i.e.* DPN, TPN, FMN, FAD) restored the activity. The inhibitory action of ammonium sulfate probably does not fully account for the inactivation observed after ammonium sulfate fractionation, since in the presence of as much as 1 mole per liter of $(NH_4)_2SO_4$ the enzyme retained 50 per cent of its activity.

RESULTS

Properties of Purified Fraction PO

The final preparation did not contain even a trace of aldehyde oxidase activity. No pyridoxal was detected in the reaction mixture when the purified preparation was incubated with pyridoxine. It is clear from Fig. 1 that the preparation itself has no ability to form pyridoxal from pyridoxine. 4-Pyridoxic acid was produced only when the rabbit liver aldehyde oxidase preparation was added initially to the incubation mixture. Moreover, the rate of pyridoxic acid formation was nearly proportional to the amount of aldehyde oxidase preparation added (Fig. 2). Aldehyde oxidase preparation had no activity to oxidize pyridoxine.

1. pH-Activity Curve—In Fig. 3 were shown pH-activity curves in the

transformation both of pyridoxine to pyridoxic acid and of pyridoxal to pyridoxic acid. The optimal activity of pyridoxine oxidation was obtained in the range from pH 5.7 to 5.9 in the presence of aldehyde oxidase prepara-

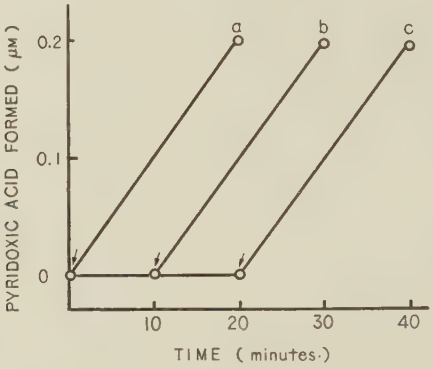


FIG. 1. Requirement for aldehyde oxidase preparation for pyridoxine oxidation.

Each tube contained, in a volume of 2.0 ml., 20 μM of pyridoxine, 1.2 mg. of the purified Fraction PO, 1 mg. of aldehyde oxidase preparation from rabbit liver and 100 μM of phosphate buffer, pH 6.0. Aldehyde oxidase was added at 0 minutes (a), 10 minutes (b) and 20 minutes (c) as indicated by the arrows.

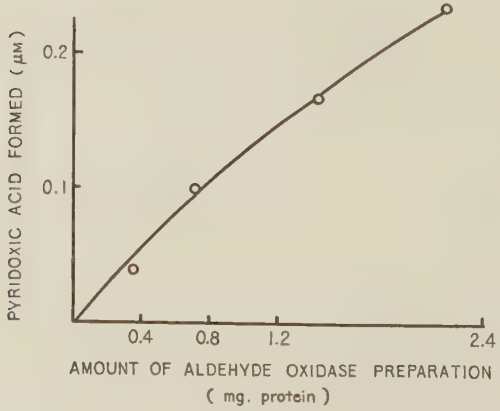


FIG. 2. Effect of amount of aldehyde oxidase preparation on the oxidation of pyridoxine.

Each tube contained, in a volume of 2.0 ml., 20 μM of pyridoxine, 1.2 mg. of the purified Fraction PO, the indicated amount of aldehyde oxidase preparation and 100 μM of phosphate buffer, pH 6.0. Reaction mixtures were incubated for 10 minutes at 37°.

tion (Curve A). On tne other hand, the oxidation of pyridoxal to pyridoxic acid by aldehyde oxidase proceeded most rapidly at a higher pH (Curve B).

The discrepancy of pH optima in the oxidation of pyridoxine and that of pyridoxal may be due to the higher rate of conversion of pyridoxine to pyridoxal in the acidic pH under the assay conditions used.

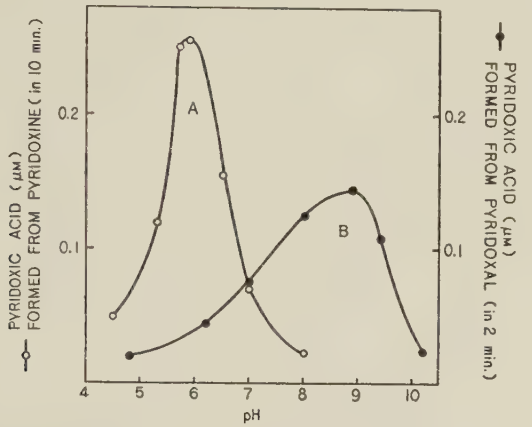


Fig. 3. Effect of pH on the reaction rates in the conversion of pyridoxine and pyridoxal to pyridoxic acid.

Curve A: incubation conditions as in Fig 2 expect that 2.0 mg. of aldehyde oxidase preparation were used.

Curve B: each tube contained, in a volume of 2.0 ml., 1.0 μM of pyridoxal, 2.0 mg. of aldehyde oxidase and 100 μM of the appropriate buffer. Reaction mixtures were incubated for 2 minutes at 37°. Buffer solutions used were acetate buffer, phosphate buffer and carbonate-bicarbonate buffer.

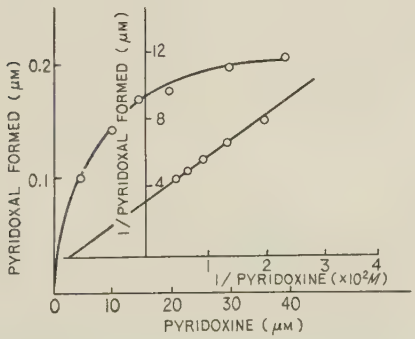


Fig. 4. Effect of pyridoxine concentration on the reaction rate.

Incubation conditions were similar to those of Fig. 2, except that the pyridoxine concentration was varied as indicated and 2.0 mg. of aldehyde oxidase preparation were used.

2. *Time Course of Reaction*—The reaction was linear with time for about 60 minutes.

3. *Effect of Fraction PO*—The enzymatic activity was nearly proportional

to Fraction PO concentration over a 5-fold range of protein.

4. Effect of Substrate Concentration –The initial reaction rates for the oxidation of pyridoxine were measured at different substrate concentrations. From these data, the Michaelis-Menten constant for the reaction has been determined from double reciprocal plot as $7.1\times10^{-3}\,M$ (Fig. 4).

5. Substrate Specificity—There was no detectable ability to oxidize pyridoxine phosphate in the final preparation. Other substrates were not examined.

6. Nature of Electron Acceptor—The addition of DPN, TPN, FAD, or FMN to the purified Fraction PO had no effect on the rate of oxidation of pyridoxine. As mentioned above, the addition of an aldehyde oxidase preparation led to the conversion of pyridoxine to pyridoxic acid. On the other hand a decrease of pyridoxal was studied with the purified preparation in the presence of DPNH or TPNH, but no decrease of pyridoxal was observed. However, the yeast enzyme was found to reduced pyridoxal to pyridoxine in the presence of TPNH at pH 7 to 8 as described later.

A pressed cake of baker's yeast was liquified by the addition of 0.3 volume of toluene for two hours at 40° and then extracted with an equal volume of 0.1 *M* phosphate buffer, pH 7.4 for thirty minutes with occasional stirring at 10°. The middle aqueous layer was separated after centrifugation at $18{,}000\times g$ for thirty minutes. 31.3 g. of solid ammonium sulfate per 100 ml. were added to it. The precipitate was discarded. An additional 6.6 g. of ammonium sulfate were added per 100 ml. of the supernatant fluid. The

TABLE II

Requirement for TPNH in the Reduction of Pyridoxal by the Yeast Enzyme Preparation

The complete reaction mixture contained, in 2.0 ml., 0.7 μM of pyridoxal, 0.3 μM of TPN, 6.0 μM of glucose-6-phosphate, glucose-6-phosphate dehydrogenase (8.4 unit), the enzyme from baker's yeast and 100 μM of phosphate buffer, pH 7.4. Incubation was carried out for 30 minutes at 37°.

Condition	Pyridoxal disappeared
Complete system	0.4 μM
−TPN	0.0
−Glucose-6-phosphate dehydrogenase	0.01
−TPN, Glucose-6-phosphate dehydrogenase	0.0

resulting precipitate was collected by centrifugation and dissolved in a minimal amount of cold distilled water. Although the final preparation contained low activities of glucose-6-phosphate dehydrogenase and TPNH-oxidase, it was used for following experiment without further purification. Glucose-6-phosphate dehydrogenase was prepared according to the method described by Kornberg *et al.* (*6*). This preparation showed a considerable activity in 0.01 *M* phosphate buffer, pH 7.4 without addition of magnesium ion.

Involvement of TPNH in the reduction of pyridoxal is shown in Table II. In this case, DPNH was without effect on the reduction of pyridoxal. Subsequently, the reduction of several artificial dyes by pyridoxine in

TABLE III

Effect of Various Metal Ions on the Enzyme Activity in the Crude Extracts

Each tube contained, in 2.0 ml., 10 μM of pyridoxine, 5×10^{-4} moles of each metal ion, 0.2 ml. of rabbit liver crude extracts and 100 μM of phosphate buffer, pH 6.0. Reaction mixtures were incubated for 10 minutes at 37°.

Addition	Pyridoxic acid formed
none	0.013 μM
Fe^{++}	0.038
Mn^{++}	0.030
Ca^{++}	0.012
Mg^{++}	0.013
Fe^{+++}	0.012
Al^{+++}	0.012
Co^{++}	0.011
Ni^{++}	0.012
Fe^{++} [1]	0.000

1) Heated extracts were used.

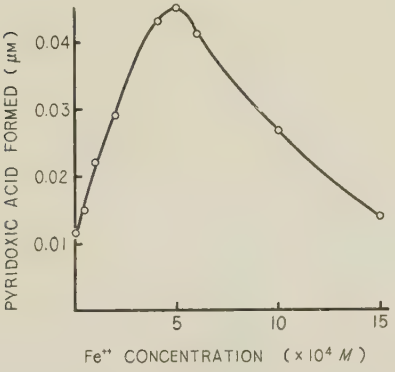


FIG. 5. Effect of Fe^{++} concentration on the enzyme activity in crude extracts.

Each tube contained, in 2.0 ml., 10 μM of pyridoxine, 0.2 ml. of the rabbit liver extract, the indicated amount of $FeSO_4(NH_4)_2SO_4\cdot6H_2O$ and 100 μM of phosphate buffer pH 6.0. Reaction mixtures were incubated for 15 minutes at 37°.

the presence of the purified Fraction PO was examined. Methylene blue, 2,6-dichlorophenolindophenol, ferricyanide and phenazine methosulfate failed

to show any reduction.

7. Activators—a) Metal ions: The pyridoxine oxidizing activity in the crude extracts was enhanced considerably in the presence of Fe^{++} and, to a lesser extent, of Mn^{++}. However, Ca^{++}, Mg^{++}, Fe^{+++}, Al^{+++}, Co^{++} and Ni^{++}

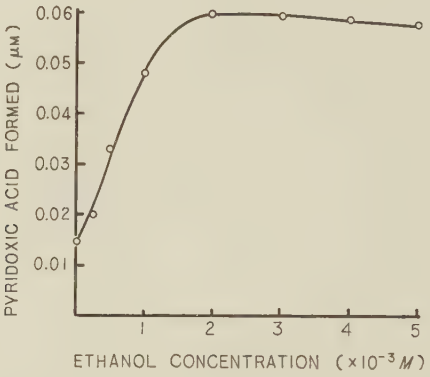


Fig. 6. Effect of ethanol concentration on the enzyme activity in crude extracts.

Each tube contained, in 2.0 ml., 10 μM of pyridoxine, indicated amount of ethanol, 0.2 ml. of rabbit liver crude extracts and 100 μM of phosphate buffer, pH 6.0. Reaction mixtures were incubated for 10 minutes at 37°.

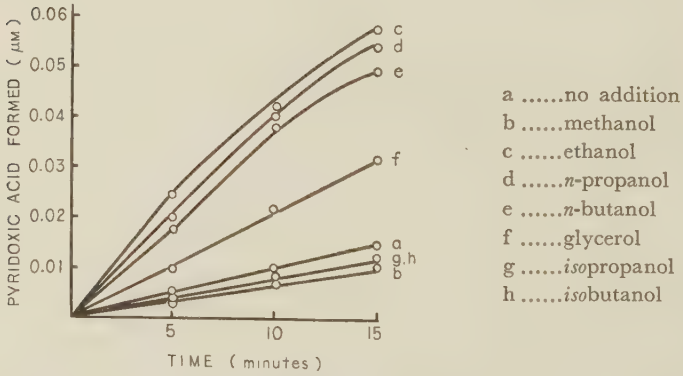


Fig. 7. Effect of various aliphatic alcohols on the enzyme activity in crude extracts.

Each tube contained, in 2.0 ml., 10 μM of pyridoxine, 2×10^{-3} moles of alcohol, 0.2 ml. of rabbit liver extracts and 100 μM of phosphate buffer, pH 6.0. Reaction mixtures were incubated for 15 minutes at 37°.

had no effect (Table III). The optimal concentration of Fe^{++} was 5×10^{-4} *M* as indicated in Fig. 5. The stimulatory action of Fe^{++} and Mn^{++} disappeared when a purified preparation was used.

b) Aliphatic alcohols: The pyridoxine oxidizing activity of the crude

extracts and of an exhaustively dialyzed purified preparation were found to be accelerated in the presence of ethanol. The relation of the ethanol concentration to the pyridoxine oxidizing activity is shown in Fig. 6. Similarly, *n*-propanol, *n*-butanol and, to a lesser extent, glycerol enhanced the activity at the same concentration (Fig. 7). The product which was formed in the

TABLE IV

Effect of Several Analogues of Pyridoxine on the Oxidation of Pyridoxine

Incubation conditions were identical with those of Fig. 2, except that each analogue of pyridoxine was added in the indicated amount to the reaction mixture.

Compound	Concentration μM	Inhibition percentage	
		Pyridoxine to 4-pyridoxic acid	Pyridoxal to 4-pyridoxic acid
None		0	0
4-Deoxypyridoxine	25	67	0
Pyridine	40	87	0
Nicotinamide	40	6	4
Nicotinic acid	40	47	40
Toxopyrimidine	40	24	6

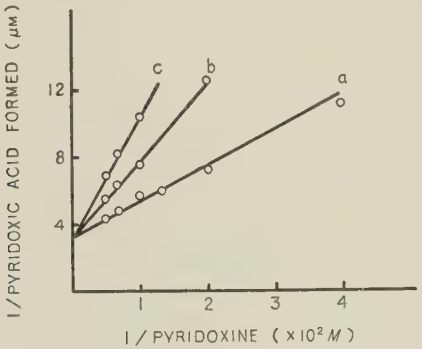


FIG. 8. Inhibition of pyridoxine oxidation by 4-deoxypyridoxine.

Incubation conditions were similar to those of Fig. 2, except that in experiment (b) and (c) 5.0 and 15.0 μM of 4-deoxypyridoxine, respectively, were added in the reaction mixtures.

presence of Fe^{++} or alcohol was identified to be 4-pyridoxic acid by use of paper chromatography. On the chromatograms the R_f value of the product coincided with that of authentic 4-pyridoxic acid. On the other hand, methanol, *iso*propanol and *iso*butanol had no activating effect (Fig. 7). The mechanism of the effect of these alcohols is quite obscure.

8. Inhibition by Analogues of Pyridoxine—4-Deoxypyridoxine had been

demonstrated to inhibit the phosphorylation of pyridoxal by pyridoxal kinase competitively. The possibility that pyridoxine analogues might be inhibitors in the oxidation of pyridoxine was examined. Among the compound tested, 4-deoxypyridoxine and pyridine showed a definite inhibitory action (Table IV). The mode of inhibition 4-deoxypyridoxine was found to be a competi-

TABLE V

Distribution of Pyridoxine Oxidizing Activity in Mammalian Tissues

Assay mixtures contained, in 2.0 ml., 25 μM of pyridoxine, 0.5 ml. of 33 % homogenate, 2.2 mg. of aldehyde oxidase preparation and 100 μM of phosphate buffer, pH 6.0, and were incubated for 30 minutes at 37°.

Tissue		Pyridoxal formed, in μM per g. of tissue in 30 minutes.
Rabbit	Liver	0.40
	Kidney	0.28
	Brain	0.28
	Spleen	0.25
	Heart muscle	0.12
	Bone marrow	0.16
	Lung	0.20
	Pancreas	0.0
	Serum	0.03
	Blood corpuscle	0.50
Rat	Liver	0.21
	Kidney	0.24

tive one as can be seen from Fig. 8. The apparent inhibition constant was calculated to be $3.3 \sim 3.6 \times 10^{-3}$ *M*.

9. Distribution of the Pyridoxine Oxidizing Activity—Table V shows that the enzyme system which oxidizes pyridoxine is widely distributed among animal tissues. The rabbit liver and blood corpuscles have the highest activity. It is interesting to note differences in the pyridoxine oxidizing activity and the pyridoxine phosphate oxidase which will be reported in the next paper.

DISCUSSION

It is very reasonable to assume pyridoxal as an intermediate in the conversion of pyridoxine to pyridoxic acid by a pyridoxine oxidizing enzyme system of rabbit liver. If so, the oxidation of pyridoxine to pyridoxic acid could be envisioned as two-step reaction and the equilibrium of the first reaction, *i.e.*, the interconversion between pyridoxine and pyridoxal, might lie far in the direction of pyridoxine formation, so that the addition of aldehyde oxidase would accelerate the conversion of pyridoxine to pyridoxal by removing the latter compound from the reaction system. Unfortunately,

any evidence to support this hypothesis has not yet been obtained with rabbit liver enzyme system. Recent findings in our laboratory, however, suggest that pyridoxine is oxidized to pyridoxal and then to pyridoxic acid in living organisms. Actually a TPN-specific pyridoxine dehydrogenase has been partially purified from extracts of baker's yeast and its reaction mechanism has been studied. The reaction was found to favor the reduction of pyridoxal at a neutral pH*.

The investigation to demonstrate in animal tissues an enzymatic activity analogous to that found in baker's yeast is now under way. The relationship between pyridoxine oxidizing enzyme system and pyridoxine dehydrogenase system is also being studied.

SUMMARY

1. The protein fraction which catalyzes pyridoxine oxidation in the presence of aldehyde oxidase preparation was purified from rabbit liver extracts.

2. The pyridoxine oxidizing enzyme system showed the highest activity at pH 5.7 to 5.9.

3. The ability of Fraction PO to oxidise pyridoxine was dependent on the presence of aldehyde oxidase preparation from rabbit liver.

4. The pyridoxine oxidizing activity in the crude extracts was enhanced by the addition of ferrous ion, manganese ion and some aliphatic alcohols. The stimulatory action of these metal ions was not observed with a purified preparation.

5. 4-Deoxypyridoxine competitively inhibited the oxidation of pyridoxine.

6. No electron acceptor in this reaction could be identified in the liver system. However, a preliminary experiment with the yeast enzyme revealed a requirement for TPNH in the conversion of pyridoxal to pyridoxine.

7. The distribution of the pyridoxine oxidizing activity was studied in several mammalian tissues.

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ENZYMATIC STUDIES ON PYRIDOXINE METABOLISM

III. PYRIDOXINE PHOSPHATE OXIDASE

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(Received for publication, November 28, 1959)

It has been recently proposed that pyridoxine phosphate might be a plausible intermediate in the conversion of pyridoxine to pyridoxal phosphate (*1*).

This paper describes data on the purification, some properties, and the reaction mechanism of an enzyme from rabbit liver which catalyzes the conversion of pyridoxine phosphate to pyridoxal phosphate. This enzyme is designated as pyridoxine phosphate oxidase on the basis of the reaction mechanism.

EXPERIMENTAL

Materials and Methods

Preparation of Pyridoxine Phosphate and 4-Deoxypyridoxine Phosphate—Calcium salts of pyridoxine phosphate and 4-deoxypyridoxine phosphate were prepared from pyridoxine hydrochloride and 4-deoxypyridoxine hydrochloride respectively, according to the method of Heyl *et al.* (*2*). The concentrations of these compounds were calculated spectrophotometrically using the value of $E_{310}^{0.1N\cdot NaOH} = 7300$ for pyridoxine phosphate and of $E_{283}^{0.1N\cdot HCl} = 8600$ for 4-deoxypyridoxine phosphate (*3*).

Pyridoxine phosphate was synthesized also by two other method; (a) Reduction of pyridoxal phosphate by sodium borohydride* and (b) Conversion of pyridoxamine phosphate to pyridoxine phosphate by nitrite (*3*).

Enzyme Assay—The reaction mixture was immersed in boiling water for one minute, and the pyridoxal phosphate formed was determined using apo-tryptophanase prepared from *E. coli* (K-12) (*4*). The preparation was shown to decompose tryptophan to indole in proportion to the amount of pyridoxal phosphate present.

Preparation of Enzyme—460 g. of liver from desanguinated rabbits were ground with quartz sand and extracted with 1380 ml. of cold distilled water. The following procedures were carried out at 0° to 5°. The supernatant obtained after centrifugation at 13,000×*g* for 20 minutes was adjusted to pH 5.0 with 1 *M* acetic acid. The resulting bulky precipitate was centrifuged off. The supernatant was brought to neutrality, and then 17.6 g. of solid ammonium sulfate per 100 ml. of supernatant solution was added. To the supernatant obtained after centrifugation, an additional 12.7 g. of ammonium sulfate per 100 ml. of the supernatant was added. After standing for 30 minutes, the precipitate was collected and dissolved in 500 ml. of distilled water. The enzyme solution thus obtained was fractionated by the addition of 99 per cent ethanol at −5° to 0°. The

* Matsuo, Y., Personal communication

precipitate formed in 50 per cent ethanol was discarded. Further addition of ethanol to give 70 per cent saturation yielded a second precipitate which was dissolved in 50 ml. of cold distilled water and dialyzed against running water in the cold overnight. The resulting precipitate was removed by centrifugation. The supernatant fluid was adjusted to pH 5.0 with 1 *M* acetic acid and calcium phosphate gel was added in the proportion of 0.6 g. of gel per g. of protein. The gel was collected and the enzyme which was adsorbed on the gel was eluted first with 20 ml. and then with 10 ml. of 0.1 *M* phosphate buffer, pH 8.0. The combined eluates were dialyzed against running water for three hours and then brought to pH 5.0. To the resulting solution, 0.2 g. of alumina Cγ gel per g. of protein was added. The enzyme was recovered from the gel by elution with 20 ml. of 0.1 *M* phosphate buffer, pH 8.0. The results of a typical purification are summarized in Table I.

TABLE I

Purification of Pyridoxine Phosphate Oxidase

Specific activity; pyridoxal phosphate formed mμM/mg. of protein/10 minutes. Each tube contained, in a volume of 1.0 ml., 0.5 μM of pyridoxine phosphate, the enzyme preparation and 50 μM of phosphate buffer, pH 8.3. Reaction mixtures were incubated for ten minutes at 37°.

	Pyridoxine phosphate oxidase
Crude extract	3.80
pH 5.0 Supernatant	4.50
0.3–0.5 $(NH_4)_2SO_4$	19.70
50–70% Alcohol fraction	83.2
Ca-gel eluate	93.0
Al Cγ eluate	189.0

RESULTS

Properties of the Purified Enzyme

1) pH Optimum—The effect of pH on the activity of pyridoxine phosphate oxidase is shown in Fig. 1.

2) Substrate Concentration—The relationship between pyridoxine phosphate concentrations and the reaction rates is shown in Fig. 2. From double reciprocal plots, the K_m value was calculated as 2.0×10^{-5} *M/L* for pyridoxine phosphate.

3) Inhibition of Pyridoxine Phosphate Oxidase by 4-Deoxypyridoxine Phosphate—In the previous paper pyridoxine oxidation was reported to be inhibited competitively by 4-deoxypyridoxine (*6*). As indicated in Fig. 3, the oxidation of pyridoxine phosphate was inhibited competitively by 4-deoxypyridoxine phosphate, but it was not inhibited by 4-deoxypyridoxine even at a concentration one thousand times as high as that of pyridoxine phosphate.

4) Electron Acceptor—The conversion of pyridoxine phosphate to pyridoxal phosphate did not proceed anaerobically. Under these conditions, on the

addition of methylene blue to the incubation mixture containing the purified pyridoxine phosphate oxidase and pyridoxine phosphate, bleaching of the

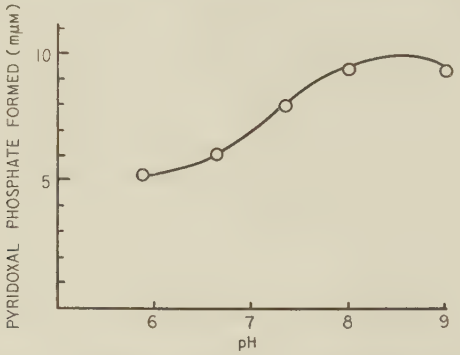

FIG. 1. Effect of pH on enzyme activity.

Each tube contained, in a volume of 1.0 ml., 500 mμM of pyridoxine phosphate, the purified enzyme and 100 μM of phosphate buffer. Reaction mixtures were incubated for ten minutes at 37°.

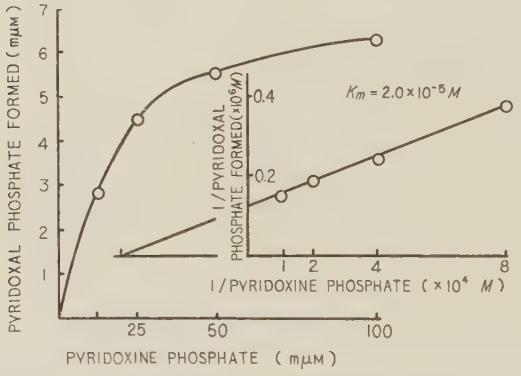

FIG. 2. Effect of pyridoxine phosphate concentration on the reaction rate.

Each tube contained, in a volume of 1.0 ml., 12.5–100 mμM of pyridoxine phosphate, the purified enzyme and 100 μM of phosphate buffer, pH 8.0. Reaction mixtures were incubated for ten minutes at 37°.

dye and formation of pyridoxal phosphate were observed (Fig. 4).

In order to resolve the prosthetic group from the enzyme, the following modification of Warburg-Christian's method was used. 10 ml. of saturated ammonium sulfate adjusted to pH 3.6 with glacial acetic acid was added dropwise to 10 ml. of the purified enzyme solution. The resulting precipitate was immediately collected by centrifugation and dissolved in 5.0 ml. of cold distilled water. The enzyme solution obtained after three repetition of this

procedure was dialyzed against 100 volumes of cold distilled water overnight. The final preparation showed very low activity, which was considerably

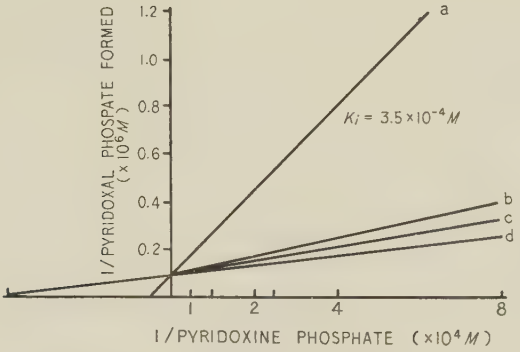

Fig. 3. Inhibition of pyridoxine phosphate oxidase by 4-deoxypyridoxine phosphate.

Each tube contained, in a volume of 1.0 ml., the indicated amount of pyridoxine phosphate, 4-deoxypyridoxine phosphate, the purified enzyme and 100 μM of phosphate buffer.

4-Deoxypyridoxine, a : 1.0 μM, b : 0.5 μM
c : 0.125 μM, d : none

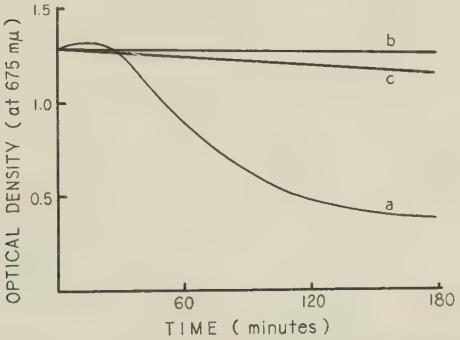

Fig. 4. Time course of the reduction of methylene blue.

In experiment a) 1 μM of pyridoxine phosphate, 250 μM sodium fluoride, 88 μg. of the purified enzyme, 1 μM of methylene blue and 100 μM of phosphate buffer, pH 8.0, were incubated at 37° in a Warburg vessel in an atmosphere of nitrogen. In experiment b), pyridoxine phosphate was omitted. In experiment c), pyridoxine phosphate was replaced by 0.1 μM of pyridoxal phosphate.

restored by the addition of FMN or FAD (Fig. 5). DPN and TPN were ineffective.

5) Stoichiometry of the Reaction—A sonic extract of *Pseudomonas aeruginosa* was found to be highly active in the oxidation of pyridoxine phosphate.

The pyridoxine phosphate oxidase from *Pseudomonas*, was quite similar to the rabbit liver enzyme in its FMN or FAD requirements and affinity for the substrate. For the stoichiometric study, the *Pseudomonas* extract was used.

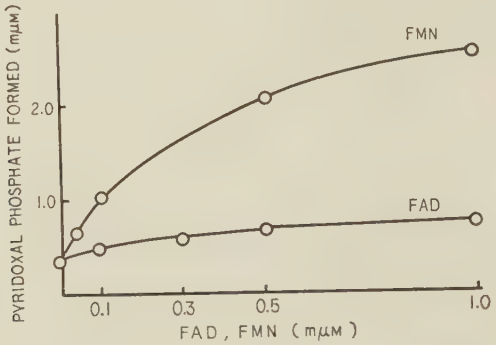

Fig. 5. Effect of FMN or FAD concentration on the oxidation of pyridoxine phosphate.

Each tube contained, in a volume of 0.8 ml., 0.2 ml. of acid ammonium sulfate-precipitated oxidase, 0.05–1.0 mμM of FAD or FMN and 100 μM of phosphate buffer, pH 8.3. Reaction mixtures were preincubated for 20 minutes at 37°, and then for 10 minutes at 37° with 0.5 μM of pyridoxine phosphate.

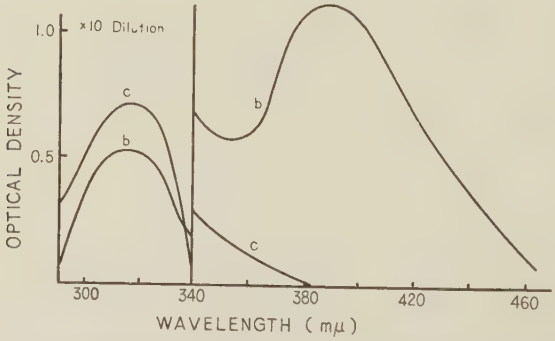

Fig. 6. Spectrograms of the same reaction mixtures as in Table II.

As indicated in Table II, 0.81 μM of pyridoxal phosphate was formed during the consumption of 0.79 μM atom of oxygen and the disappearance of 0.83 μM of pyridoxine phosphate.

The concentrations of pyridoxine phosphate and pyridoxal phosphate were read from the spectrograms of the reaction mixtures (Fig. 6).

When 60 μM of ethanol were added to the reaction mixture, oxygen consumption approximately doubled (Fig. 7). This is characteristic of a flavine enzyme.

6) *Substrate Specificity*—The purified enzyme had activity to oxidize

pyridoxine to pyridoxal. No other substrates except pyridoxine were examined. Preparations of pyridoxine phosphate synthesized by two other methods, as described in 'Experimental', also reacted with the purified enzyme to form pyridoxal phosphate.

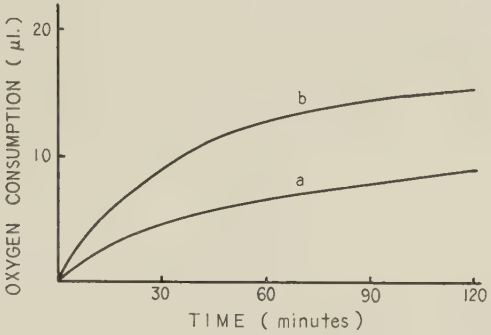

Fig. 7. Time course of oxygen consumption in the conversion of pyridoxine phosphate to pyridoxal phosphate.

In experiment (a), 4.2 μM of pyridoxine phosphate, 2.0 ml. of a sonic extract[1] of *Pseudomonas aeruginosa* and 150 μM of phosphate buffer, pH 8.3, were incubated at 37° in a Warburg vessel under an atmosphere of oxygen. In experiment (b), 60 μM of ethanol were used.

1) The extract contained a high activity of catalase.

Table II

Stoichiometry of Pyridoxine Phosphate Oxidation (Pseudomonas)

Experiment	Ethanol[1]	Oxygen[2] uptake	Pyridoxine[3] phosphate decreased	Pyridoxal[3] phosphate formed
a) *Pseudomonas* enzyme + pyridoxine phosphate	−	μ atom 0.84	μM 0.83	μM 0.81
b) *Pseudomonas* enzyme + pyridoxine phosphate	+	1.56	0.82	0.80
c) Pyridoxine phosphate	−	0.05	0.0	0.0
d) Pyridoxine phosphate	+	0.06	0.0	0.0

1) 60 μM for vessel.
2) calculated from the data of Fig. 7.
3) read from the spectrogram of Fig. 6.

7) Distribution of Pyridoxine Phosphate Oxidase and Pyridoxamine Phosphate Oxidase—Table III shows the distribution of pyridoxine phosphate oxidase, along with that of pyridoxamine phosphate oxidase (*6*) in various tissues of rabbit and rat. It is of some interest to note that the activities of these two oxidases are confined to a few tissues, contrary to a broader distribution of

Table III

Pyridoxine Phosphate and Pyridoxamine Phosphate Oxidase Activity of Mammalian Tissues

Assay mixtures contained, in a volume of 1.0 ml., 0.2 μM of pyridoxine phosphate or pyridoxamine phosphate, 150 μM of sodium fluoride, 0.2 ml. of a ten per cent homogenate and 50 μM of phosphate buffer, pH 8.4. Reaction mixtures were incubated for ten minutes at 37°.

Tissue	Pyridoxine phosphate oxidase activity	Pyridoxamine phosphate oxidase activity
	μM/g.	μM/g.
Rabbit liver	0.32	0.325
kidney	0.06	0.07
brain	0.025	0.025
spleen	0.01	0
heart muscle	0	0.015
bone marrow	0	0
lung	0	0
pancrease	0	0
serum	0	0
blood corpuscle	0.06	0.02
Rat liver	0.215	0.12
kidney	0.08	0.065

Table IV

Formation of Pyridoxal Phosphate in Streptoccus faecalis

Each tube contained, in 1.0 ml., 0.5 ml. of a sonicated cell suspension of *Streptococcus faecalis* and 100 μM of phosphate buffer, pH 5.3. Reaction mixtures were incubated for thirty minutes at 37°.

Substrate	Pyridoxal phosphate formed	Pyridoxine phosphate formed[1]
	mμM	mμM
1. Pyridoxine (0.15 μM)+ATP (3 μM)+Mg^{++} (0.5 μM)	0	1.53
2. Pyridoxal (0.15 μM)+ATP (3 μM)+Mg^{++} (0.5 μM)	1.78	
3. Pyridoxine phosphate (0.15 μM)	0.00	

1) After deproteinization of the reaction mixture, a 0.5 ml. aliquot was incubated with purified liver oxidase at pH 8.4 to convert pyridoxine phosphate to pyridoxal phosphate. From the amount of pyridoxal phosphate formed and a standard curve for the conversion of pyridoxine phosphate to pyridoxal phosphate by liver oxidase, the initial amount of pyridoxine phosphate was calculated.

the enzyme which oxidizes pyridoxine.

8) Absence of Pyridoxine Phosphate Oxidase from Streptococcus faecalis R—

Streptococcus faecalis R grows well on a medium containing pyridoxal or pyridoxamine as a source of vitamin B_6 but slowly on a medium containing pyridoxine instead of pyridoxal or pyridoxamine (*7*). To see the reason for this, the activities of the kinases for pyridoxine, pyridoxal, and pyridoxine phosphate oxidase of *Streptococcus faecalis* were examined using cells which were ruptured by sonic oscillation. Pyridoxine phosphate formed from pyridoxine on the addition of ATP was measured after conversion to pyridoxal phosphate by subsequent incubation with a pyridoxine phosphate oxidase preparation from rabbit liver which was free from kinase activity. Table IV indicates that *Streptococcus faecalis* has no pyridoxine phosphate oxidase activity.

DISCUSSION

Previously it was suggested that, in the conversion of pyridoxine to pyridoxal phosphate, pyridoxine phosphate oxidase plays an important role and pyridoxine phosphate is a plausible intermediate in living organisms (*1*). The K_m value of pyridoxine phosphate oxidase for pyridoxine phosphate (2.0×10^{-5} *M*) (*6*) is much smaller than that of the enzyme oxidizing pyridoxine (7.1×10^{-3} *M*). The higher apparent affinity of pyridoxine phosphate oxidase for its substrate fortifies the proposition that pyridoxine phosphate oxidase is a key-enzyme in the formation of pyridoxal phosphate. This will be verified by the demonstration of pyridoxine phosphate in natural sources. This study is now in progress. Pyridoxine phosphate seems to exist in the baker's yeast.

The antagonistic action of 4-deoxypyridoxine to pyridoxine had been illustrated as follows: 4-deoxypyridoxine inhibits not only the phosphorylation of pyridoxine and pyridoxal but also the combination of pyridoxal phosphate with apo-enzyme, after being converted to 4-deoxypyridoxine phosphate. In the previous paper, 4-deoxypyridoxine was shown to inhibit the oxidation of pyridoxine to pyridoxal and the above data have revealed that 4-deoxypyridoxine phosphate competitively inhibits the oxidation of pyridoxine phosphate to pyridoxal phosphate.

The prosthetic group of pyridoxine phosphate oxidase seems to be FMN rather than FAD. The result of stoichiometric study supports the following reaction mechanism;

$$\text{Pyridoxine phosphate}+O_2\longrightarrow\text{pyridoxal phosphate}+H_2O_2$$

Growth curve of *Streptococcus faecalis* R in various media are consistent with the results obtained from the assay of enzyme activity involved in the metabolism of, at least, pyridoxine. The influence of cell membrane permeability should also be borne in mind.

Several other microorganisms have been known to show different requirements for pyridoxine, pyridoxal or pyridoxamine as a source of vitamin B_6. Results of nutritional experiments with them may answer the problem when the activities of kinase, pyridoxamine phosphate oxidase, pyridoxine phosphate oxidase *etc.*, are studied in each of microorganism.

SUMMARY

1. Pyridoxine phosphate oxidase was purified from a rabbit liver extract.
2. The oxidase was most active at pH 8.0 to 9.0.
3. 4-Deoxypyridoxine phosphate competitively inhibited the oxidation of pyridoxine phosphate.
4. The oxidase was resolved from its prosthetic group by treatment with acidic ammonium sulfate. The apo-oxidase showed higher affinity for FMN than for FAD.
5. Stoichiometry of the reaction was studied in a sonic extract of *Pseudomonas aeruginosa*. The result supported the following reaction mecanism:

$$\text{Pyridoxine phosphate} + O_2 \longrightarrow \text{Pyridoxal phosphate} + H_2O$$

6. The distribution of the enzyme activity was studied in several mammalian tissues and *Streptococcus faecalis*. In the latter pyridoxine phosphate oxidase seems to be absent.

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TURNIP PEROXIDASE

III. THE PHYSICOCHEMICAL PROPERTIES OF CRYSTALLINE TURNIP PEROXIDASE D

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(Received for publication, December 18, 1959)

Among the three kinds of turnip peroxidases described previously (*1*), only turnip peroxidase D was obtained in a crystalline form and its activity was the highest as evident from the values of purpurogallin number and the rate constant of the reaction between Complex II and hydrogen donor (*1*, *2*). Thus, it was considered to be of interest to study more extensively the physicochemical properties of the peroxidase, and to compare them with those of other known peroxidases. In this paper, the results obtained through the measurements of absorption spectrum, sedimentation and diffusion are described.

RESULTS AND DISCUSSION

The preparation of peroxidase used throughout the present work was the recrystallized turnip peroxidase D prepared by the method described in the first paper of this series (*1*).

Absorption Maxima of Several Derivatives of Turnip Peroxidase D and the Dissociation Constant of Hydrogen Ion from the Water Molecule Linked to the Iron Atom of Hematin—The absorption spectra of the compounds of the peroxidase with HF, HCN, HN_3 and CO were measured according to procedures similar to those of Keilin and Hartree (*3*). The wavelength of absorption maxima and corresponding millimolar extinction coefficients obtained are summarized in Table I. The resulting values resemble those observed in horseradish peroxidase (*4*). Accordingly, the previous view (*1*) that turnip peroxidase D may have the same prosthetic group as that of horseradish peroxidase has been further confirmed.

The absorption spectrum of alkaline peroxidase was measured in a manner similar to that of Morita and Kameda (*5*). The absorption spectrum was found to be rather unstable as pH increased beyond 11.5. It may be due to the alkali-splitting of hematin from the peroxidase molecule, in view of the fact that such splitting was observed in the case of horseadish peroxidase at above pH 12.5 (*6*). The isosbestic points between the spectra of neutral and alkaline peroxidase were found at about 367, 460, 550 and

635 mμ.

The alkaline peroxidase is considered to be formed by the release of a hydrogen ion from the water molecule linked to the iron of hematin (*3*). The pK value of the dissociation was determined spectrophotometrically at 580 mμ in a manner similar to that of Morita and Kameda (*5*). The values of absorbance at pH above 11.5 were carefully read immediately after the addition of potassium hydroxide, taking into consideration of the fact mentioned above. The pK value obtained was 10.28 at ionic strength of 0.1 and at 23°, lying between the values for horseradish peroxidase (*7*) and Japanese-radish peroxidase *a* (*5*), *i.e.* 10.91 and 9.57, respectively.

TABLE I

Wavelengths (mμ) of Absorption Maxima and Corresponding Millimolar Extinction Coefficients in Parenthesis of Derivatives of Turnip Peroxidase D

Compound with	Soret band		Visible bands		
HF		404 (107.5)	490 (10.0)	540s (7.55)	613 (4.58)
HCN		420 (107.5)		542 (11.5)	
HN_3		414 (102.1)		534 (8.45)	635 (1.97)
Red.+CO		422		543	572.5
OH^-	360	414		540	578

's' signifies shoulder.

Diffusion Coefficient—Diffusion experiments were made at 25.0° in a Neurath-type diffusion cell (*8*) equipped with a Philpot-Svensson's shlieren optical system (*9*). The solution of the peroxidase submitted to the experiment was 0.6 per cent in the protein concentration in phosphate buffer of pH 7.05 and ionic strength 0.2. The average values of D_M and D_A calculated from the data of four exprosures from 21,600 to 28,860 sec. were found to be 8.37×10^{-7} cm^2. sec^{-1}. and 8.11×10^{-7} cm^2. sec.$^{-1}$ respectively, where D_M is the diffusion coefficient (corrected to the value in water at 20°) calculated by means of the "moment method", and D_A the coefficient calculated by of the "maximum ordinate-area method" (*cf.* Gralén (*10*)). A homogenous protein should give the same value for D_M and D_A. A non-homogenous protein should give $D_M > D_A$. The present results gave D_M slightly higher than D_A, but the difference may be within the limits of experimental error. Thus we are justified in saying that the present diffusion experiment indicates the homogeneity of the material. From the analysis of the diffusion pattern obtained, it appeared that the diffusion coefficient is independent of the protein concentration. Hence, the value of D_M obtained was taken for the calculation of molecular weight and frictional ratio.

Sedimention Coefficient—The method of sedimentation measurement was the same as that of the previous paper (*1*) except that the measurement of the

rotor temperature was made with a rotor temperature indicating unit. The sedimentation experiments* of the peroxidase preparation were carried out in three concentrations of the enzyme. The ultracentrifugal patterns showed always one symmetrical boundary, indicating again the homogeneity of the preparation. Assuming a partial specific volume of 0.75 or 0.70 for the protein, the calculation of the sedimentation coefficients ($s_{20,w}$) was made. It was based on the fact that most proteins have a partial specific volume of about 0.75 (*11*), but that the value of horseradish peroxidase was found to be 0.690 (*12*) or 0.699 (*13*). The values obtained were found to be independent of the peroxidase concentration, as shown in Table II. Accordingly, the average values were taken as the sedimentation constant at infinite dilution in the following calculations.

TABLE II

Sedimentation Coefficients of Turnip Peroxidase D

The measurements were carried out in phosphate buffer of pH 7.05 and ionic strength 0.2.

Protein concentration (%)	Temp. in Measurement (°C)	s observed (S)	$s_{20,w}$ (S)	
			I[1]	II[2]
0.24	20.2	3.30	3.53	3.51
0.40	19.4	3.30	3.63	3.58
0.51	18.8	3.25	3.60	3.57
			mean 3.58	mean 3.55

1) The partial specific volume is assumed to be 0.75.
2) The partial specific volume is assumed to be 0.70.

Molecular Weight, Frictional Ratio and Axial Ratio—Combining the values of the sedimentation and diffusion constants, unhydrated molecular weight was calculated by the aid of the formula deduced by Svedberg and Pedersen (*14*) to be 41,500 or 34,300 on the basis of the partial specific volume of 0.75 or 0.70, respectively. Both, especially the former, are in good agreement with the value of the minimum molecular weight, 43,000, which was calculated from the value of hematin content (*1*). Accordingly, it is concluded that turnip peroxidase D contains one molecule of hematin per one molecule of the enzyme as in the case of horseradish peroxidase (*4*) and Japanese-radish peroxidase *a* (*15*).

The frictional ratio, f/f_0, was also calculated using the values of the sedimentation and diffusion constants and listed in Table III. By the use of these values, the axial ratio of the molecule was also calculated according to the procedures of Perrin (*16*), assuming the molecular shape to be prolate ellipsoid or oblate ellipsoid. The results obtained are also listed in Table III.

* For this measurement the author is indebted to Mr. O. Tarutani of our laboratory.

TABLE III

Molecular Weight and Shape of Various Peroxidases

Peroxidase	Prosthetic group	Molecular weight	Partial specific volume[1] (ml./g.)	Frictional ratio	Axial ratio		Method	Reference
					as prolate ellipsoid a/b	as oblate ellipsoid b/a		
Horseradish peroxidase	Ferriproto-porphyrin	41,200	0.690	1.39	7.4	8.4	Sedimentation & diffusion	(*4*, *12*)[2]
		39,800	0.699	1.36	6.7	7.6	Sedimentation & diffusion	(*13*)
Japanese-radish peroxidase *a*	Ferriproto-porphyrin	55,700	(0.75)	1.22	4.5	4.9	Osmotic pressure & diffusion	(*15*)
Turnip peroxidase A_1	Ferriproto-porphyrin	49,000					Chemical analysis	(*1*)
Turnip peroxidase A_2	Ferriproto-porphyrin	45,000					Chemical analysis	(*1*)
Turnip peroxidase D	Ferriproto-porphyrin	43,000					Chemical analysis	(*1*)
		41,500	(0.75)	1.10	2.9	3.0	Sedimentation & diffusion	The present paper
		34,300	(0.70)	1.23	4.6	5.0	Sedimentation & diffusion	The present paper
Cytochrome *c* peroxidase	Unknown heme	60,000					Chemical analysis	(*17*)
Myeloperoxidase	Unknown heme	56,700					Chemical analysis	(*18*)
Lactoperoxidase	Unknown heme	92,700	0.764	1.18	4.0	4.2	Sedimentation & diffusion	(*19*)
		82,000					Light-scattering	(*20*)

1) The values in parenthesis are ones assumed.

2) The values of this row have been recalculated by the presented author using $D_{20,w}=6.84\times10^{-7}$ cm^2. sec.$^{-1}$ (*12*) and $s_{20,w}=3.59$ S (*4*). The molecular weight obtained by Maehly (*4*), 40,200, may be erroneous.

It is of interest to compare these values of turnip peroxidase D with the corresponding values of other kinds of purified peroxidases, which are taken from reports by other workers in this field and also tabulated in Table III. As evident from this table, the molecular weight of turnip peroxidase D is very close to that of horseradish peroxidase, these two being the smallest among those of all the peroxidases presented in the table. However, the shape of the molecule of turnip peroxidase D may be said to be somewhat different from that of horseradish peroxidase, in that it is more spherical than that of the latter in view of the values of axial ratio of these peroxidases. In fact, the values of the axial ratio of turnip peroxidases D obtained assuming a value of 0.75 for the partial specific volume are the smallest ones in this table. Moreover, it can be shown by the procedure of Svedberg and Pedersen (*14*) that they reduce to 1.00 if the molecule is assumed to be hydrated 0.25 g. per 1 g. of protein. On the other hand, with the assumption of the partial specific volume to be 0.70, the axial ratio of the enzyme is found to become 1.00 if the hydration of 59 per cent is assumed. It is generally accepted that proteins are hydrated in the degree of 0.3-0.6 g. of water per 1 g. of protein (*21*). Thus, from the above consideration, it may be said that the shape of turnip peroxidase D is nearly spherical in solution.

SUMMARY

1. The absorption spectra of crystalline turnip peroxidase D were measured for its alkaline form and for its compounds with carbon monoxide, cyanide, azide and fluoride. They were found to agree very closely with those of horseradish peroxidase. The pK value of the dissociation of a hydrogen ion from the water molecule linked to the iron atom of hematin was found to be 10.28 at an ionic strength of 0.1.

2. The diffusion constant ($D_{20,w}$) of the enzyme was found to be 8.37×10^{-7} $cm^2 . sec^{-1}$.

3. The sedimentation constant ($s_{20,w}$) of the enzyme was found to be 3.53 S or 3.55 S, assuming the partial specific volume to be 0.75 or 0.70, respectively.

4. Using the above values, the molecular weight was calculated to be 41,500 or 34,300 and the frictional ratio 1.10 or 1.23, respectively.

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IMMUNOCHEMICAL STUDIES OF INSULIN

I. ENHANCEMENT OF NEUTRALIZING ANTIBODY FORMATION AGAINST INSULIN BY LIPOIDAL SUBSTANCES DERIVED FROM TUBERCLE BACILLI

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Recently (*1*,*2*,*3*), it was well established that the administration of certain bacterial bodies, notably tubercle bacilli, together with protein antigen results in significantly enhanced antibody level as compared to those obtained when protein antigen alone is employed. The mixture containing heat killed tubercle bacilli is known as complete Freund's adjuvant (*4*).

In our preliminary report (*5*), it was found that the immunization with insulin in complete Freund's adjuvant to guinea pig caused significant enhancement of insulin neutralizing antibody formation as compared to that obtained without tubercle bacilli.

In the present paper, the effective substance as adjuvant in tubercle bacillary bodies have been studied.

EXPERIMENTALS

Preparation of Antiinsulin Sera—The insulin preparation for immunization was made according to the modified method of Moloney and Coval (*6*) by addition of heat killed tubercle bacilli or bacillary substances as follows.

Ten ml. of a solution of insulin (Novo's Ultra Lente, 40 units/ml.), adjusted to pH 2.8 with HCl and containing 1 per cent phenol, was added to the mixture of 20 ml. of paraffin oil, 10 g. of anhydrous lanolin and 100 mg. of heat killed human type tubercle bacilli ($H_{37}R_v$) or lipoidal substances of bacilli. Emulsification was effected by rapid mechanical stirring in a mortar. Guinea pigs were immunized by subcutaneous injection of 2 ml. of antigen preparation into each of two separated areas of back region. Total dose of insulin injected was 20 units. The injection of this dose was repeated four weeks later.

Blood samples were collected by cardiac puncture three times at interval of two weeks after the first injection in order to test the insulin neutralizing activity.

Preparation of Lipid Fractions of Tubercle Bacilli—Heat killed human type tubercle bacilli ($H_{37}R_v$) were kindly supplied by Dr. Yamaguchi, Research Laboratory of the National Sanatorium, Toneyama Hospital.

Purified Wax, Wax C, Wax D, bound lipid, alcohol-ether soluble lipid and mycolic acid were prepared according to the method described by Asselineau and Lederer

(7) and proteolipid by the method of Yamamura (8). Nitrogen and phosphorus content of Wax fractions and bound lipid were as follows. Purified Wax: N 0.15 per cent P 0.18 per cent, Wax C: N 0.00 per cent, P 0.02 per cent, Wax D: N 1.6 per cent, P 0.31 per cent, bound lipid: N 1.1 per cent, P 0.47 per cent.

Buffered Saline—Phosphate buffer (0.15 *M*, pH 7.4) was combined with an equal volume of 0.9 per cent NaCl (6).

RESULTS

Neutralization of Insulin by Immune Serum from Guinea Pig—The neutralizing activity of immune serum from guinea pig was assayed by mouse convulsion procedure. The animals used were starved ddN strain. Test was carried out by injecting serum or dilutions of serum in buffered saline mixed with 0.25 units of whale insulin which, when injected with buffered saline or normal serum, caused convulsion in all of mice.

As shown in Table I, immune serum could prevent mice from the hypoglycaemic symptoms due to insulin, though no neutralizing activity was ever observed with normal guinea pig sera or buffered saline. The immune sera tested here have a neutralizing activity of 2–4 units insulin per ml.

TABLE I

Neuralization of Insulin by Guinea Pig Immune Serum Observed by Mouse Convulsion Test

Serum[1)]	ml.	Buffered saline	ml.	Number of mice showing convulsion in a group of 4 mice
Immune serum	1.0	0		0
	0.5	0.5		0
	0.25	0.75		0
	0.125	0.875		0
	0.063	0.937		4
	0.031	0.969		4
Normal serum	1.0	0		4
	0	1.0		4

Guinea pig immune serum was prepared using ox insulin as an antigen.[1)] Serum or serum diluted with buffered saline (pH 7.4) was mixed with 0.2 ml of saline containing 0.25 units of whale insulin. Separate groups of 4 mice were used for each mixture. The mice were injected subcutaneously with 1.2 ml. per animal.

Neutralization of insulin by immune serum was demonstrated not only by the mouse convulsion test but also by change in blood glucose level as seen in Fig. 1 (curve a). Normal guinea pig serum had only slight neutralizing activity (curve b). It was evident from Fig. 1 and Table I that the neutralizing activity determined by mouse convulsion test was approximately equal to those calculated by the change of blood glucose level, using the

same sample. Therefore, insulin neutralizing activity of immune serum was hereafter measured by mouse convulsion test.

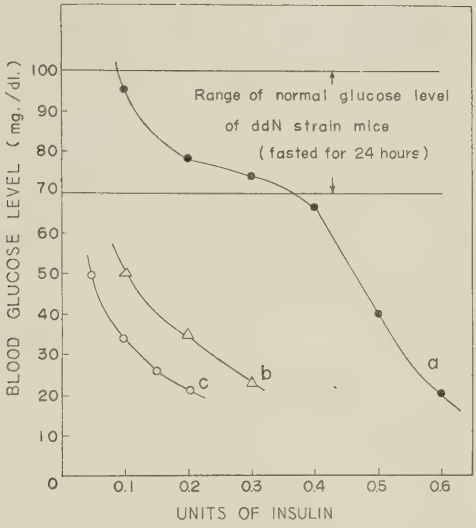

FIG. 1. Neutralization of insulin by guinea pig immune serum observed by changes of blood glucose level in mice.

a. 0.12 ml. of immune serum plus whale insulin.
b. 0.12 ml. of normal serum plus whale insulin.
c. insulin alone.

A mixture of 0.12 ml. of serum and varying dose of insulin (0.1–0.6 units) was injected subcutaneously to starved mouse. Blood sugars were determined 2 hours after injection.

Adjuvant Effect of Whole Tubercle Bacillary Bodies—The immune serum which was obtained by injection of antigen mixed with heat killed tubercle bacilli, could neutralize about 3–6 units of insulin per ml. as shown in Table II. The most potent sera showed a neutralizing activity of 25 units of insulin per ml. (No. 3 guinea pig). In contrast to these results, it was shown that immune sera obtained by the injection of antigen without heat-killed tubercle bacilli, had low neutralizing activity of only 0.5–0.75 units of insulin per ml. This adjuvant effect of tubercle bacilli could not be replaced by other microorganism, for example, by *Hemophilus pertussis* as seen in Table II.

Adjuvant Effect of Lipoidal Substance of Tubercle Bacillary Bodies—The effective substance in heat killed tubercle bacilli was investigated. Antigen preparation was made by addition of each lipid fraction instead of whole bacillary bodies, using the same dose.

As shown in Table III, the striking effect caused by whole tubercle bacillary bodies seemed to be due to mainly Wax D fraction. Proteolipid and

purified Wax were also active but other lipids from the same bacterium were found to lack this ability.

TABLE II

Enhencement of Production of Insulin Neutralizing Antibodies in Guinea Pig by Heat Killed Tubercle Bacilli

Adjuvant	Guinea pig	Insulin neutralizing activity of immune serum (unit per ml.)		
		Weeks after first injection		
		2 weeks	4 weeks	6 weeks
Without tubercle bacilli	No. 9	0–0.25	0–0.25	1–1.5
	No. 10	0	0	0.5–1
	No. 11	0	0	0
	No. 12	0–0.25	0	0.25–0.5
With tubercle bacilli	No. 1	1–2	4–8	6–8
	No. 2	0.25–0.5	1–2	3–4
	No. 3	0.5–1	2–4	6–8 (25–30)[1]
	No. 4	0.5–1	0.5–1	1–2
	No. 5	1–2	1–2	4–6
	No. 6	0.5–1	2–4	2–4
	No. 7	0.25–0.5	2–4	1–2
	No. 8	0.5–1	2–4	1–2
With *Hemophilus pertussis*	No. 48		0	0.5–1
	No. 49	0	0	0.25–0.5
	No. 50	0–0.25	0.25–0.5	0.5–1
	No. 51	0–0.25	0–0.25	0.25–1

The composition of antigen preparation was as follows.

10 ml. of insulin (Novo Ultra Lente 40 u./ml.), 20 ml. of paraffin oil, 10 g. of anhydrous lanolin, 100 mg. of heat killed tubercle bacilli.

Guinea pigs were immunized as descrived in the text. At interval of 2 weeks blood samples were taken from animals by cardiac puncture and assayed by mouse convulsion procedure.

1) Titre in parenthesis shows the activity 10 weeks after first injection. This animal (No. 3) recieved the third injection 8 weeks after first one.

TABLE III

Enhancement of Production of Insulin Neutralizing Antibodies in Guinea Pig by Various Lipids Extrable from Heat Killed Tubercle Bacilli

Fraction	Guinea pig	Insulin neutralizing activity of immune serum (units per ml)		
		Weeks after first injection		
		2 weeks	4 weeks	6 weeks
Purified Wax	No. 21	0	0–0.25	2–4
	No. 22	0–0.25	0–0.25	3–4
	No. 23	0–0.25	0.25–0.5	2–4
Wax C	No. 26	0–0.25	0–0.25	0.25–0.5
	No. 27	0	0	0.5–1
	No. 28	0–0.25	0–0.25	0.25–0.5
	No. 29	0–0.25	0	
Wax D	No. 31	0–0.25	4–6	12–16
	No. 32	0–0.25	2–4	6–8
	No. 33	0	0.25–0.5	0.5–1
	No. 34	0	0.5–1	2–4
	No. 35	0.25–0.5	1–2	1–2
Proteolipid	No. 36	0.5–1	0.5–1	0.25–0.5
	No. 37	1–2	0.5–1	1–2
	No. 38	0.25–0.5	0.5–1	0.5–1
Mycolic acid	No. 41	0–0.25	0–0.25	0.25–0.5
	No. 42	0–0.25	0	0–0.25
	No. 43	0	0	0–0.25
Alcohol-ether soluble lipid	No. 61	0	0–0.25	0–0.25
	No. 62	0	0.25–0.5	0.25–0.5
	No. 63	0	0.25–0.5	0.25–0.5
Bound lipid	No. 67	0.5–1	0.5–1	0.25–0.5
	No. 68	0.25–0.5	0–0.25	0
	No. 69	0	0.25–0.5	0.25–0.5

The composition of antigen preparation was as follows.

10 ml. of insulin (Novo Ultra Lente 40 u./ml.), 20 ml. of paraffin oil, 10 g. of anhydrous lanolin, 100 mg. of each lipid fraction.

Immunization of guinea pigs and test for the neutralizing activity of blood samples are as same as Table II.

DISCUSSION

The antibodies to neutralize the physiological action of insulin have been

reported by Lowell (*9–11*), Banting, Franks and Gairns (*12*), Marsh and Haugaard (*13*), Rausch-Stroomann and Sauer (*14*) and Arquilla and Stavitsky (*15*, *16*). However, it was difficult to obtain the clearly demonstrable insulin neutralizing antibodies in animal. This difficulty is considered to be owing to the following reasons. Firstly, insulin is weakly antigenic, presumbaly because of its low molecular weight and its less specificity, as is well known from the fact that there are only slight differences among the amino acid sequences of various insulin preparations from the different animals, ox, pig, whale, sheep and horse. Secondly, the injection with considerable amounts of insulin cause often the death of animals to be immunized due to hypoglycemic shock by insulin.

Moloney and Coval (*6*) have successfully demonstrated that insulin neutralizing antibodies could be induced in guinea pig and sheep by the injection of insulin in incomplete Freund's adjuvant. It was evident from their reports (*6*, *17*) and our results (Table II) that the titre of the antibodies was not so high without tubercle bacilli in adjuvant. The immunization of guinea pig with insulin in complete Freund's adjuvant caused significant enhancement of insulin neutralizing antibody formation.

Recently, it was reported that the ability of the tubercle bacilli to produce either the tuberculin type hypersensitivity (*1*, *2*) or experimental allergic encephalomyelitis (*3*) depends on the content of a certain lipopolysaccharide of tubercle bacilli. The present study show in agreement with these results that Wax D, lipopolysaccharide isolated from tubercle bacilli, is effective in enhancement of insulin neutralizing antibodies production. Proteolipid, which was isolated from tubercle bacilli and identified as the cavity inducing substance by Yamamura (*8*), is also active and this fraction as well as Wax D contains a large amount of mycolic acid. Raffel, Lederer and Asselineau (*18*) have shown already that galactose- and methyl-ester of mycolic acid were effective in producing of tuberculin type hypersensitivity in guinea pigs. It is suggested from these facts that the derivatives of mycolic acid must play the important role concerning the adjuvant effect of tubercle bacilli, though free mycolic acid itself is inactive.

SUMMARY

1. The antibodies responsible for insulin neutralization were induced in guinea pigs by injection of insulin in complete Freund's adjuvant.

2. The adjuvant effect of tubercle bacilli on production of neutralizing antibodies for insulin seems to depend mainly on Wax D fraction.

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PARTICULATE NITRATE REDUCTASE OF AZOTOBACTER VINELANDII*

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Multi-functional nature of microbial nitrate reduction can now be understood by classifying it essentially into two types (*1*, *2*). The first type—"nitrate respiration type"—can be found in the group of facultative anaerobes such as *E. coli*. *E. coli* enzyme has been shown to be a particulate enzyme functioning in participation with cyt. b_1*** (*3*, *4*). Recently, the enzyme solubilized and homogeneously purified has been clarified to be a new metalloprotein binding molybdenum and iron without any significant content of bound flavin (*5*). The second type—"nitrate assimilation type" is found in the group of aerobes such as *Neurospora*. *Neurospora* enzyme has been shown to be a soluble molybdoflavoenzyme functioning without any participation of cytochromes (*6*).

In *Azotobacter vinelandii*, the inducible presence of soluble pyridine nucleotide-nitrite and hydroxylamine reductases of metalloflavoprotein nature was reported by Spencer *et al.* (*7*). The particulate pyridine nucleotide-hydroxylamine reductase was also demonstrated by Bulen (*8*) in the same bacterium. However, the studies on *Azotobacter* nitrate reductase have remained very scanty.

The present paper describes the inducible presence of pyridine nucleotide-nitrate reductase of particulate and metalloenzyme nature which may be apprently classified under the nitrate assimilation type.

MATERIALS AND METHODS

Microorgamism—Azotobacter vinelandii 0—strain was grown in Burk's medium to which

* A part of this work was reported at the Annual Meeting of the Biochemical Society of Japan in 1959 at Osaka.

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*** The following abbreviations are used: cyt., cytochrome; DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; FAD and FMN, flavin adenine dinucleotide and flavin mononucleotide; Nb, nile blue; NbH_2, reduced Nb; EDTA, ethylenediamine tetraacetic acid; PCMB, *p*-chloromercuribenzoate.

was added 2.0 g KNO_3 per liter at 30° for 15–20 hours with vigorous aeration.* The time of cell harvest seemed to be critical and the cells older than 20 hours were devoid of detectable enzyme. Cells were harvested and three times washed with 0.2 per cent KCl by centrifugation to remove nitrite accumulated in the growth medium in appreciable quantity. The yield of cells under these conditions was 5 to 10 g. wet weight per liter.

*Preparation and Centrifugal Fractionation of Cell-free Extracts***—The cell-free extracts were prepared by grinding in a cold mortar for 30 minutes the fresh cell paste with 1.5 times their weight of alumina powder (Wako No. 800) and for another 10 minutes with five times their weight of cold 0.2 per cent KCl, and were obtained as the supernatant of $2{,}000\times g$ for 20 minutes. After centrifugal fractionation of the cell-free extracts at $14{,}000\times g$ for 20 minutes and at $105{,}000\times g$ for 60 minutes (preparative Spinco centrifuge Model L) at below 5°, three fractions—the large particles ($2{,}000\sim14{,}000\times g$), the small particles ($14{,}000\sim105{,}000\times g$) and soluble supernatant were obtained. The sedimented particles were washed once with ice-cold 0.2 per cent KCl, and finally suspended in 0.005 *M* phosphate buffer, pH 6.0. The suspension of large particles which was used as the enzyme preparation throughout this work could be stored at 0° for about two days without remarkable loss of activity.

Enzyme Assay

DPNH-Nitrate Reductase—This was termed for the enzyme system participating in the electron transfer from DPNH to nitrate. The activity was assayed by using Thunberg tubes with the reaction mixture contained 0.05 *M* phosphate buffer, pH 6.7, 1.5 μmoles DPNH, 30 μmoles KNO_3 (these in main tube) and the enzyme preparation (0.2∼0.6 mg. N) (in side tube) in a final volume of 3.0 ml. Prior to starting the reaction by mixing, air had to be removed perfectly from the tubes, since the enzyme preparation contained active DPNH oxidase as stated later. After the reaction for 10∼30 min. at 30° a suitable amount of neutralized $CdSO_4$ solution was added and with the deproteinized clear supernatant solution the amount of nitrite produced was measured colorimetrically in a usual manner (*4*).***

Sometimes, activity was assayed spectrophotometrically using Thunberg tube-type cuvettes. The optical density at 340 mμ was followed by Hitachi Electrospectrophotometer Model ERB-U for a control cuvette without DPNH. DPNH oxidized due to nitrate reductase activity was then given by the total decrease in DPNH minus the decrease in the absence of nitrate.

NbH_2-Nitrate Reductase—The activity utilizing NbH_2 as an electron donor was assayed in the similar manner to DPNH-nitrate reductase assay except that DPNH was replaced by 5.0 μmoles nile blue chemically reduced by H_2 gas and palladium. When the effect of various donors was studied, NbH_2 was replaced by other dyes reduced by small amounts of dithionite less than 1 mg.

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* Cells grown on N_2 gas or ammonium sulfate were obtained essentially in the same manner as described above except that the nitrogen source was air or ammonimum sulfate (0.94 g. per liter) respectively instead of KNO_3. When ammonimum sulfate was used, $CaCO_3$ (0.5 g. per liter) was added to avoid a lowering of the pH of the medium.

** The procedures employed were essentially similar to that described by Rose *et al.* (*9*).

*** Since DPNH interferes with the development of azo dye in the determination of nitrite produced in DPNH-NaR reaction, the reaction mixture, prior to the addition of $CdSO_4$ solution, was vigorously shaken and left to stand in a open tube at 30° for about 5 minutes to oxidize remaining DPNH by means of DPNH oxidase in the particles.

DPNH-Nitrite and Hydroxylamine Reductases—These activities were assayed under the conditions similar to those of DPNH-nitrate reductase assay except that nitrate was replaced by each substrate of 3.0 μmoles and 20 μM FAD was further added to the reaction mixture. The decreased amounts of substrate were obtained by subtracting from the amounts of each zero time control. Hydroxylamine was determined according to the procedures employed by Kono *et al.* (*10*).

Aerobic Disappearance of Nitrate—The activity was assayed at 30° by measuring the decreased amounts of nitrate in the following reaction mixture contained *Azotobacter* cell suspension (0.2~0.5 mg. N) (in side room), 0.08 *M* phosphate buffer. pH 7.1, 100 μmoles glucose, 30 μmoles KNO_3 in a final volume of 3.0 ml. in Warburg manometer vessels with shaking under air phase. After the reaction for 30 minutes, appropriate portion of the reaction mixture was immediately diluted by cold distilled water and centrifuged in the cold. The nitrate content in the clear supernatant obtained was determined by the modified method described by Mullin *et al.* (*11*). The decreased amounts were obtained by subtracting from the amounts of each zero time control.

DPNH and Succinate Oxidases—The assay conditions employed were essentially similar to those described by Bruemmer *et al.* (*12*). and by Rose *et al.* (*9*), respectively.

Units and Specific Activites of Nitrate, Nitrite and Hydroxylamine Reductases, DPNH and Succinate Oxidases—One unit of these enzymes was defined as the amount required to produce 1.0 μmole of nitrite, to result in the disappearance of 1.0 μmole of nitrite or hydroxylamine, to oxidize 1.0 μmole of DPNH, or to take up 1.0 μatom of oxygen for one hour, respectively. Specific activities of these were expressed as units per mg. of Kjeldahl nitrogen in enzyme preparation.

Cytochrome Observation—Cytochrome spectra were examined by an ocular spectroscope (Abbe's spectral ocular Zeiss). As a reducing reagent, sodium dithionite was used. The difference spectra were followed by Hitachi Electrospectrophotometer Model ERB-4.

Materials—DPNH and TPNH were prepared by enzymatic reduction of each nucleotide of 70 per cent or more purity by the method of Pullman *et al.* (*13*) and by Evans *et al.* (*14*), respectively. FAD and FMN of about 80 per cent purity was kindly donated by Dr. J. Okuda. 2-Hydroxy-3-(2-methyloctyl)-1.4-naphthoquinone (SN 5949), 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) and antimycin A were kindly donated by Drs. R. W. Estabrook, R. Sato and T. Asahi and used according to details described by Clark *et al.* (*15*), Lightbown *et al.* (*16*) and Potter *et al.* (*17*), respectively.

RESULTS

Distribution of Nitrate Reductase Activity after Centrifugal Fractionation of Cell-free Extracts—As illustrated in Table I, nitrate reductase activity has been shown to be abundantly concentrated in the large particles regardless of the electron donors. No significant effect of the addition of the soluble supernatant or small particles on DPNH-nitrate reductase activity of the large particles was confirmed. It was also found that almost all of activities of succinate dehydrogenase and hydrogenase assayed by using methylene blue as an electron acceptor resided in the same particles.

Spectroscopic examination of cytochromes revealed that the large particles bound cytochrome components including cyt. c_4, c_5 (these two cytochromes apparently yeielded a single combined α band), b, a_1 and a_2 whereas some of these cytochromes could not be detected in small particles and in soluble

supernatant. Absence of DPNH-nitrite reductase activity in the large particles was confirmed. DPNH-nitrite reductase of significant specific activity (0.5–0.9) was, on the contrary, found only in the soluble supernatant among

TABLE I

Distribution of Nitrate Reductase Activity after Centrifugal Fractionation of Cell-free Extracts of Azotobacter vinelandii

Fraction	N (mg.)	Nitrate reductase activity			
		from DPNH		from reduced nile blue	
		Untis	Specific activity	Units	Specific activity
Cell-free extract (2,000×*g*)	25	4.8	0.19	12.6	0.50
Large particles (2,000~14,000×*g*)	10	4.3	0.43	11.4	1.14
Small particles (14,000~105,000×*g*)	6	0.7	0.12	1.9	0.32
Soluble supernatant (105,000×*g*<)	8	0.5	0.06	1.4	0.17

2.0 g. wet weight of cells was used. For the detailed conditions see text.

three fractions.* On the other hand, DPNH-hydroxylamine reductase activity was found in the large particles (spectific activity of about 0.2) as well as in the soluble supernatant with the higher specific activity of 0.4–0.6.

Electron Donors Specificity for Particulate Nitrate Reductase and Effect of Externally Added Flavin—The stimulatory effect of DPNH and TPNH on the rate of nitrate reduction is shown in Fig. 1. A small amounts of reduction

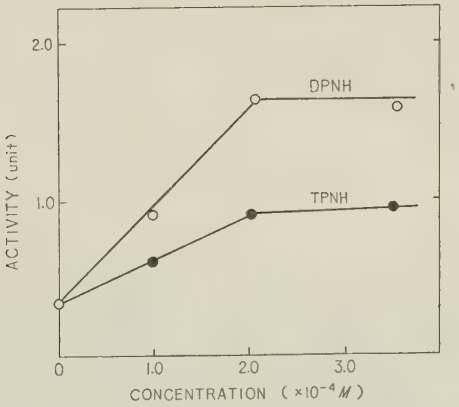

FIG. 1. Effect of concentration of reduced pyridine nucleotides as electron donors on *Azotobacter* nitrate reductase activity.
For the detailed conditions see text.

* The possibility that apparently very low DPNH-nitrate reductase activity of the soluble supernatant is due to the further reduction of nitrite by DPNH-nitrite reductase in the same fraction can be excluded by showing no decrease of nitrate by the determination of nitrate.

occurred without the addition of reduced pyridine nucleotides were increased about 4-fold in the presence of DPNH. TPNH was less effective. Succinate was sometimes shown to serve as an electron donor but the activity frequently fluctuated.

In the standard assay for DPNH-nitrate reductase, the reaction was followed simultaneously either by the appearance of nitrite or spectrophotometrically by the disappearance of DPNH. As one example of such experimental results, 0.120 μmole DPNH has been oxidized (corrected for minus nitrate control) and 0.100 μmole NO_2^- formed (corrected for minus DPNH control) indicating the stoichiometrical relationship according to the following equation: $NO_3^- + DPNH + H^+ \rightarrow NO_2^- + DPN^+ + H_2O$.

The DPNH-nitrate reductase activity was stimulated 1.5- to 2-fold by the external addition of saturated level (20 μM) of FAD or FMN.

The rates of nitrate reduction were compared among saturated level of reduced form of redox dyes. Table II shows that reduced forms of nile blue,

Table II

Effect of Reduced Redox Dyes as Electron Donors for Azotobacter Nitrate Reductase

Redox dyes reduced by dithionite	Units
None	0.04
2,6-Dichlorophenol indophenol	0.08
Thionine	0.10
Methylene blue	0.10
Toluidine blue	0.38
Nile blue	0.44
Phenosafranine	0.40
DPNH[1)]	0.20

1) Enzymatically reduced

For the detailed conditions see text.

toluidine blue and phenosafranine were effective whereas those of methylene blue, thionine and 2,6-dichlorophenol indophenol were much less effective. DPNH could be replaced by the reduced form of nile blue resulting in about 2-fold increase of the reduction rate. Unlike the DPNH-nitrate reductase activity, NbH_2-nitrate reductase activity was never stimulated by the addition of even high level (100 μM) of FAD or FMN.

The Proportionality between Enzyme Concentration and Initial Rate of Nitrate Reducton—This proportionality was shown to be established within the range of about 1.5 units under the standard assay conditions for DPNH- and NbH_2-nitrate reductase.

Effect of pH on Nitrate Reductase Activities—As shown in Fig. 2, maximal activities for the both systems were obtained using 0.05 M phosphate buffer at around pH 6.7.

Substrate Saturation—The relationship between nitrate concentrations and NbH_2-nitrate reductase activities is shown by the Lineweaver-Burk's

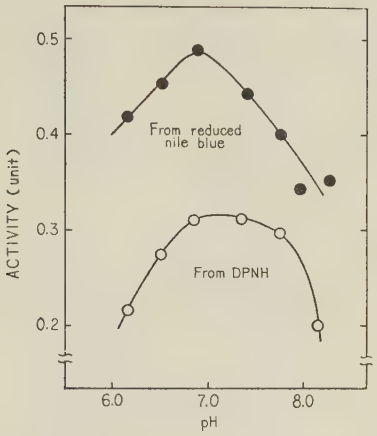

Fig. 2. Effect of pH on *Azotobacter* nitrate reductase activities.
For the detailed conditions see text.

reciprocal plot in Fig. 3. The Michaelis constant for the nitrate-enzyme complex was estimated to be $5.3\times10^{-4}\,M$.

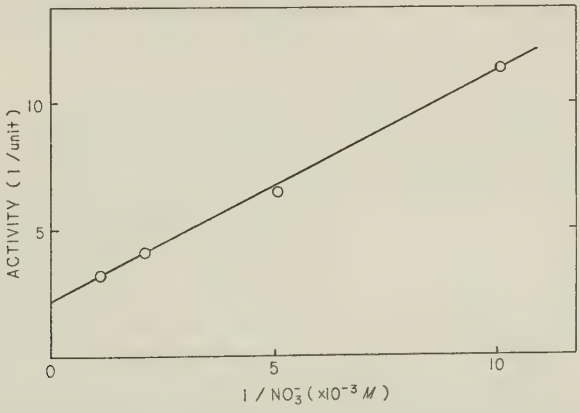

Fig. 3. Effect of nitrate concentrations on *Azotobacter* reduced nile blue-nitrate reductase activity.
For the detailed conditions see text.

Effect of Inhibitors—The effect of a range of inhibitors on DPNH- and NbH_2-nitrate reductases activities is shown in Table III. Cyanide and azide resulted in an appreciable inhibition of the both activities whereas CO (1 atm. in the dark) did not, indicating a CO-insensitive heavy metal constituent in a common terminal enzyme. The sensitivity of DPNH-nitrate reductase

to *o*-phenanthroline, EDTA, amytal, quinine and PCMB was never seen for NbH_2-nitrate reductase activity. The inhibition produced by PCMB on

TABLE III

Effect of Inhibitors on Azotobacter Nitrate Reductase Activity

Inhibitor	Final molarity	Inhibition per cent	
		from DPNH	from reduced nile blue
KCN	10^{-3}	90	85
	10^{-4}		20
NaN_3	10^{-3}		90
	10^{-4}	75	80
CO (dark)	1 atm.	0	0
o-phenanthroline	10^{-2}	60	0
EDTA	10^{-3}	40	0
Amytal	10^{-3}	55	0
Quinine	10^{-3}	60	0
Dicumarol	10^{-3}	0	0
PCMB	5×10^{-4}	75	0
PCMB($5\times10^{-4}M$)+ glutathione ($10^{-3}M$)		20	

Inhibitors were mixed with reaction mixture at zero time. No inhibitory effect was produced by thiourea, salicylaldoxime (each in 10^{-3} *M*), Tiron, diethyldithiocarbamate (each in 2×10^{-3} *M*) and dithizon (5×10^{-3} *M*).

DPNH-nitrate reductase was effectively restored by the addition of glutathione. These results indicate that a metallic activator, flavin component and -SH group may possibly participate in the activity of DPNH-nitrate reductase but never in that of NbH_2-nitrate reductase. DPNH-oxidase activity of the large particles was shown to be not inhibited by the same level of *o*-phenanthroline or EDTA. Sensitivity of DPNH-nitrate reductase to amytal and quinine may correspond to the stimulatory effect of FAD or FMN specifically produced on DPNH system.

DPNH- and NbH_2 activities were not inhibited even by high level of cytochrome inhibitors, HOQNO, SN 5949 and antimycin A at final concentration of 6.0, 10.0, and 15.0 μg. per ml., respectively. Insensitivities of DPNH-oxidase of the large particles to antimycin A (10.0 μg. per ml.) was also confirmed.

Functioning of Cytochromes -The large particles were shown to contain cytochrome components such as cyt. c_4, c_5 (singly combined α-band), b, a_1 and a_2. The difference spectum (reduced by DPNH minus oxidized) of the particles observed anaerobically is recorded in Fig. 4. Besides a peak at 428 mμ in the Soret region, peaks were observed at 525,552 and 630 mμ and two humps were obtained at 560 and around 595 mμ, respectively. Peaks at

525 and 552 mμ may due to the combined reduced β- and α-peaks of cyt. c_4 and c_5, respectively. A peak at 630 mμ suggests the presence of cyt. a_2. Cyt.

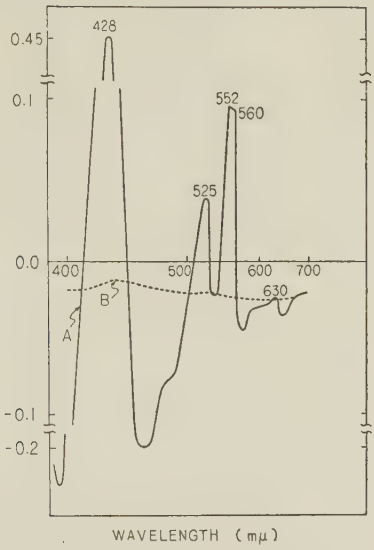

FIG. 4. Difference spectra of *Azotobacter* particles.
curve A; (reduced by DPNH for 30 min. anaerobically)-(oxidized)
curve B; (reduced form by DPNH was reoxidized by air for 5 min.)-(oxidized)
The cuvette for the reduced form contained 0.05 *M* phosphate buffer, pH 6.7, 5.0 μmoles DPNH and *Azotobacter* particles of 1.5 mg. N. in a final volume of 3.0 ml. anaerobically in Thunberg tube-type cuvette. The reference cuvette for oxidized form contained same components except DPNH in an open cuvette.

b_1 may be associated with the hump at 560 mμ. Broad hump around at 595 mμ may correspond to the α-absorption of cyt. a_1 (*18*). The whole character of the differece spectrum accords to the previous results of spectroscopic observation. No significant change of the difference spectrum by the addition of 10 μmoles solid KNO_3 and followed incubation for 30 minutes was caused under anaerobic conditions, while signifirant formation of nitrite during the incubation was confirmed. On the contrary, the remarkable change was caused by introduction of air and the difference spectrum shown as curve A shifted rapidly to curve B in Fig. 4 after 3 minutes from the introduction indicating that all cytochrome components reduced by DPNH were completely oxidized by molecular oxygen. The results indicate that these cytochrome components participate not in the electron-transferring system from DPNH to nitrate but in that from DPNH to oxygen.

Inducible Nature of Azotobacter Nitrate Reductase—The nitrate reductase activities of the cell-free extracts from *Azotobacter* cells grown in media in which ammonium sulfate or N_2 replaces KNO_3 as the sole nitrogen soure are recorded in Table IV. The data of Table IV demonstrated that nitrate

Table IV

The Effect of Nitrogen Source on the Formation of Azotobacter Nitrate Reductase

Fraction	Nitrogen source	N(mg.)	Units of activity	
			from reduced nile blue	from DPNH
Cell-free extract	KNO_3	25	4.8	0.2
	N_2	20	0.0	0.0
	$(NH_4)_2SO_4$	25	0.0	0.0

2.0 g. wet weight of each cells was used.

reductase activities were only detected in the extracts of nitrate grown cells indicating the inducible nature of the enzyme.

Oxidative Activities of the Large Particles from Nitrate Grown Azotobacter Cells—In order to compare the DPNH-nitrate reductase activity with oxidases activities of the particles, DPNH and succinate oxidases activities of the particles from *Azotobacter* cells grown on three different nitrogen sources were studied.

Table V

Oxidative Activities of the Particles from Azotobacter Cells Grown on Nitrate, Ammonium Sulfate, or N_2 as Sole Nitrogen Source

Nitrogen source	Specific activities of oxidases	
	for DPNH	for succinate
KNO_3	450	59
N_2	900	148
$(NH_4)_2SO_4$	—	138

Both DPNH and succinate oxidases activities of the particles from nitrate grown cells were less than those of particles from cells grown on other nitrogen sources, however, these oxidase activites of the particles from nitrate grown cells were still far stronger than activity of the DPNH-nitrate reductase.

Aerobic Disappearance of Nitrate by Azotobacter Cells—In connection with the high level of respiratory activities of *Azotobacter* particles, the aerobic and much active system metabolizing nitrate was found. This system can be

distinguishable from DPNH-nitrate reductase of anaerobic system which was shown to be poisoned by oxygen and of the lower activity. The active enzymatic disapperance of nitrate by *Azotobacter* cells with simultaneous oxidation of glucose is recorded in Table VI. As seen from the same table, nitrite

TABLE VI

Aerobic Nitrate Disappearance by Azotobacter Cells

Reaction system	Decreased NO_3^- (μmoles)/hr. mg. N
Complete	37.0
-Glucose	6.6
boiled cells	1.7
in open tube without shaking (semi anaerobic)	0.0
in Thunberg tube (anaerobic)	0.0
Complete (for nitrite as substrate)	7.0[1]

1) Decreased NO_2^- (μmoles)/hr. mg. N.

was also shown to disappear aerobically under the same conditions. The extensive studies on the products of the aerobic metabolism are now under progress. The activity of aerobic nitrate disappearance of nitrate grown cells was about 2 times higher than that of N_2 grown cells. Only about 10–20 per cent of the activity of the aerobic system in living cells has been found in the cell-free extracts before described. This activity has not yet been more successfully extracted in a cell-free state.

DISCUSSION

Pyridine nucleotide-nitrite and hydroxylamine reductases of which large portions were shown to localize in the soluble cytoplasmic fraction from *Azotobacter* in accord with the results of Spencer *et al.* (*7*). On the contrary, nitrate reductase of 90 per cent of that in the cell-free extracts was confirmed to reside in the large particles obtained between 2,000–14,000$\times g$

In contrast to the large particles corresponding to that studied by Rose *et al.* (*9*) on the activity of oxidative phosphorylation, small particles similar to that characterized by Tissieres *et al.* (*19*) to possess much higher activities of respiration and oxidative phosphorylation exhibited to have poor units of nitrate reductase activity.

Cytochrome components bound in the large particles were shown to participate not in DPNH-nitrate reductase but in DPNH oxidase activity. This accords with Sato's observation (*20*) by means of sensitive spectrophotometric method that the reduced steady state of the cytochromes in the living cells of the same bacterium remained and was not affected by the nitrate reduction.

The electron transport sequence leading to nitrate resided in the large particles from nitrate grown *Azotobacter* cells can be summarized as the fol-

lowing scheme. In addition to the flavin component, possible participation of some metallic ion and -SH group in the sequence from DPNH to nitrate reductase was suggested.

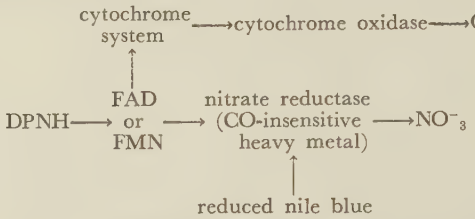

FIG. 5. Particulate nitrate reductase of *Azotobacter*.

The respiratory activities for DPNH and succinate of the large particles were shown to be remarkably supressed by culturing the cells on nitrate as sole nitrogen source. Similar effect of nitrate on hydrogenase activity was reported (*21*). However, specific activities of DPNH-nitrate reductase of the large particles from nitrate grown cells were far lower than that of the DPNH oxidase of the same particles. The situations are quite different from those of the particulate formate-nitrate reductase from anaerobically grown *E. coli* of nitrate respiration type (*5*). In the case of *E. coli*, the specific activity of the formate-nitrate reductase has been shown to be much higher than that of the formate oxidase. From these considerations, it seems to be improbable that the present DPNH-nitrate reductase may perform a physiologically significant function of the respiration in place of DPNH oxidase under anaerobiosis. In view of the independency from cytochrome systems and furthermore, of the presence of nitrite and hydroxylamine reductase in the same cells, the present system may be tentatively classifyed under the nitrate assimilation type in spite of its particulate nature and remarkable sensitivity to oxygen.

The aerobic system metabolizing nitrate found in *Azotobacter* cells was shown to be far more active than the anaerobic system suggesting more direct participation of the aerobic system in nitrate assimilation of this aerobe. Presumably, the aerobic system clearly distinguishable from anaerobic system in respect to oxygen effect will be a functional system *in vivo*, though the data here available are still too scanty to prove this with certainty. The aerobic system in *E. coli* cells similar to that described here has been studied in details (*1*, *22*). In *E. coli* system, the occurrence of nitrite or ammonia as reduction products were confirmed. The physiological and enzymatic interrelation between the anaerobic and aerobic system as well as the characterization on enzyme levels of the latter system remains to be eluidated.

SUMMARY

1. The inducible presence of DPNH-nitrate reductase of sulfhydryl CO-insensitive metalloenzyme nature in the large particles from nitrate grown *Azotobacter* cells was described. The activity was stimulated 1.5- to 2-fold by

externally added FAD or FMN. Small activity of DPNH-hydroxylamine reductase was also found to reside in the same particles, while DPNH-nitrite reductase activity was never found.

2. Reduced nile blue was shown to be a most effective electron donor with 2- to 3-fold increase in the reaction rate from that of DPNH-nitrate reductase. The reduced nile blue-nitrate reductase activity was unaffected by FAD, FMN and *p*-chloromercuribenzoate.

3. In contrast to DPNH oxidase in the large particles, DPNH-nitrate reductase has no participation of cytochrome components bound to the particles, though the latter system was inhibited by oxygen.

4. The scheme of electron-transferring sequence leading to nitrate apparently of nitrate assimilation type was presented.

5. The particulate nitrate reductase had *Km* of $5.3\times10^{-4}\,M$ for enzyme-nitrate complex and pH optimum at 6.7.

6. The presence of pyridine nucleotide-nitrite and hydroxylamine reductase of significant activites in the soluble cytoplasmic fraction was confirmed.

7. DPNH and succinate oxidases activities of the large particles were suppressed by culturing the cells on nitrate as a sole nitrogen source, these were, however, still far stronger than DPNH-NaR activity.

8. An aerobic system metabolizing nitrate and nitrite with simultaneous oxidation of glucose was found in nitrate grown *Azotobacter* cells. The aerobic system is far more active than the anaerobic one suggesting a more direct participation of the aerobic system in nitrate assimilation of this aerobe.

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CHEMICAL MODIFICATION OF TUBERCULIN PROTEIN

I. DINITROPHENYLATION AND PHENYLAZOBENZOYLATION

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Since Koch's discovery of tuberculin, various attempts have been made to isolate its active principle. A number of reports (*1-8*) have indicated that the main active principle is of protein nature. Various proteins, *e.g.* A, B, C, PPD, π, *etc.*, possesing tuberculin activity have been isolated. Recently, the chemical modification of biologically active proteins has often been attempted to determine the structure responsible for their biological activities and to prepare their specific derivatives for comparative purposes. Takeya and Mifuchi (*9*) reported that the amino groups in a tuberculin-active protein named π may be responsible for the activity. Ishida (*10*) found that several amino group blocking reagents as well as various oxidizing and reducing treatments had no effect on the activity of a tuberculin-active protein isolated from Citrate-tuberculin. Okamoto and coworkers (*11*) prepared *o*-aminophenol azo tuberculin from culture media of several strains of tubercule bacilli and suggested that tyrosyl and/or histidyl groups may not be essential for its activity. It was demonstrated by Kato (*12*) and Uragami (*13*) that the treatment of various tuberculin-active proteins with iodine or bromine resulted in a rapid inactivation of the tuberculin potency. Inspite of these findings described above, no satisfactory explanations have not yet been advanced for the correlation between the chemical structure of tuberculin-active proteins and their biological activity.

The present investigation was also undertaken to elucidate the essential groups of a tuberculin-active protein by modifying it with chemical reagents which are known to react with difinite groups of proteins. 2,4-Dinitrofluorobenzene (DNFB) and *p*-phenylazobenzoyl chloride (PABC) were chosen as the reagents because the number of these reagents introduced could be determined relatively easily.

The results obtained in the present investigation seem to suggest that half of the lysine residues and the phenol group of tyrosine residues are not essential for the tuberculin activity.

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EXPERIMENTAL

MATERIALS

Tuberculin Protein—The tuberculin protein was prepared according to the method described by Seibert (*1*) with slight modifications.

The *tubercle bacillus, human strain Aoyama-B*, was grown on Sauton's synthetic medium for six weeks. The medium was sterilized by heating at 100° for 30 minutes. The cells were removed by filtration through a layer of Hyflo Supercel. The filtrate was concentrated by means of ultrafiltration to one twentith of the original volume. The tuberculin-active protein was precipitated from the concentrated filtrate by acidification to pH 3.5–4.0 with 2 *N* HCl, centrifuged, washed with dilute acid, and then purified by redissolving in 0.05 *M* Na_2HPO_4 and reprecipitating with dilute acid. The precipitate was dissolved in 0.05 *M* Na_2HPO_4. An equal volume of saturated ammonium sulfate solution was added to the solution and the resulting precipitate was filtered with suction. After this precedure was repeated, the precipitate was dissolved in dilute alkaline solution and then dialyzed against distilled water for two days. The tuberculin protein, designated as hC, was precipitated from the dialysate by acidification to pH 3.5, washed with dilute acid, alcohol and ether.

The protein, which was light hoary powder, contained 15.8 per cent nitrogen. No sugar was detected by means of the Molisch reaction. The ultraviolet absorption spectrum of hC in 0.1 *N* NaOH is shown in Fig. 1.

0.05 μg. of hC is as potent as 0.1 ml. of *Old Tuberculin* ($\times$2000 dilution) as seen in Table II.

Chemical Reagents—PABC was kindly supplied by Dr. K. Sugae, Osaka University. All other chemicals were purchased commercially.

METHODS AND RESULTS

Biological Testing—One tenth ml. each of the solution to be tested and *Old Tuberculin* ($\times$2000 dilution) was injected intracutaneously into the same person simultaneously. Skin reaction was measured at 24 and 48 hors after the injection. In Table II and III are recorded only the 48 hour measurings.

Dinitrophenylation of hC with DNFB—Two methods have been developed for the preparation of dinitrophenyl hC (DNP-hC).

Method I. Twenty ml. of methanol containing 0.2 ml. of DNFB was added with stirring to 300 mg. of hC dissolved in 130 ml. of 0.05 *M* Na_2HPO_4 and the mixture was allowed to stand at room temperature for 20 hours. On acidification of the reaction mixture to pH 3.0 with 2 *N* HCl, yellow material was precipitated. The precipitate was centrifuged, washed with diluted acid, and dissolved in 50 ml. of 0.05 *M* Na_2HPO_4. A small amount of insoluble materials was removed by filtration. The filtrate was adjusted again to pH 3.0. This procedure was repeated two times and the precipitate finally formed was washed with alcohol until the supernatant solution became colourless, and then dried with ether. The product thus obtained and designated as DNP-hC-S was yellow powder. The yield was about 290 mg.

DNP-hC-S is soluble in neutral and alkaline solution but insoluble in acid solution. The absorption spectrum of DNP-hC-S in 0.1 *N* NaOH is

shown in Fig. 1. The absorption maxima were observed at 260 mμ and 350 mμ.

Method II. Three hundred mg. of hC was also dinitrophenylated by treating a solution of hC in 10 per cent $NaHCO_3$ with a twice volume of alcohol containing DNFB according to the method of Sanger (*14*). The yield was about 280 mg. The product designated as DNP-hC-I. DNP-hC-I is insoluble in alkaline as well as in acid solution.

Chromatographic technique was applied to quantitatively estimate the

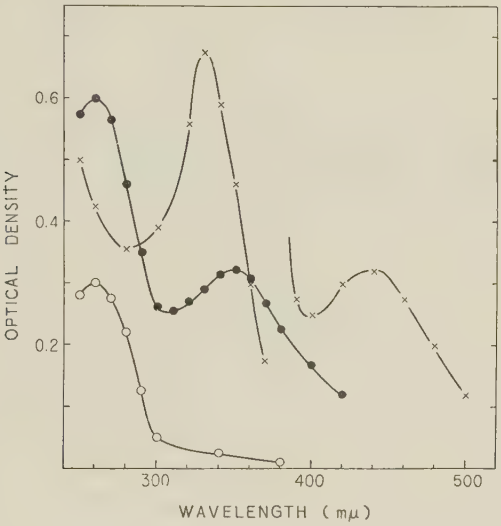

Fig. 1. Absorption spectra of hC, DNP-hC-S and PAB-hC in 0.1 *N* NaOH.
—○— hC, 0.1 mg. per ml., —●— DNP-hC-S, 0.1 mg. per ml., —×— PAB-hC, 0.1 mg. per ml. from 250 to 370 mμ, 1.0 mg. per ml. from 370 to 500 mμ.

extent of reaction of DNFB with hC as described in the following section.

Determination of ε-DNP-Lysine and O-DNP-Tyrosine in DNP-hC-S and DNP-hC-I—In order to compare the products obtained by the two different dinitrophenylation methods described above, the contents of ε-DNP-lysine and O-DNP-tyrosine in the DNP-hC's were determined.

The method was similar to that of Seki (*15*) except that a mixture of 2.5 *N* HCl and CH_3COOH (3:1) was used as eluent and that a shorter column was employed. Both DNP-hC-S and DNP-hC-I were hydrolyzed with 6 *N* HCl in a sealed glass tube for ten hours at 110°. After removal of the excess of HCl *in vacuo*, the residual hydrolysate was taken up in definite volume of the mixture of 2.5 *N* HCl and CH_3COOH. The hydrolysate corresponding to about 5 mg. of original protein was charged on a column of purified Amberlite IR-112, H-form (0.9×8.5 cm), and developed with eluent under gravity or sufficient pressure to give a flow rate of about 2 ml. per hours. The eluates were collected in 2 ml. fractions with an automatic fraction collector and the DNP-amino acids were estimated by measuring of

absorbancy at 300 mμ (for O-DNP-tyrosine) and at 360 mμ (for ε-DNP-lysine).

A typical elution curve is presented in Fig. 2, and the analytical values,

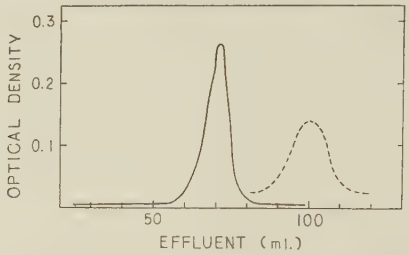

Fig. 2. Elution curve of ε-DNP-lysine and O-DNP-tyrosine. Sample; Hydrolysate of 3.7 mg. of DNP-hC-I. Column; Amberlite-IR-112, 0.9×8.5 cm. Eluent; 2.5 *N* HCl : CH_3COOH (3 : 1). —— Optical density measured at 360 mμ. ----- measured at 300 mμ.

Table I

Contents of Lysine, Tyrosine and Their DNP-Derivatives in hC and Two DPN-hC

	Residues of amino acid per 10^5 g. protein		
	hC	DNP-hC-S[1]	DNP-hC-I[1]
ε-DNP-Lys		9.1	14.6
O-DNP-Tyr		11.8	14.2
Lys	24.8[2]	15.7[3]	10.2[3]
Tyr	14.0[2]	2.2[3]	0[3]

1) The amount of original hC in the DNP-hC was determined by estimation of the amide content of the both proteins.
2) Determined by ion exchange resin chromatography (*17*).
3) Calculated values.

which are averages of duplicate determinations, are shown in Table I. The correction factor, 1.1, was applied to both DNP-amino acids (*16*).

Tuberculin Activity of DNP-hC—

DNP-hC-S. As seen in Table II, DNP-hC-S was as potent as unmodified hC. This fact suggests that the dinitrophenylation caused no alterations of the structure around the active site.

DNP-hC-I. Since DNP-hC-I is insoluble in usual aqueous solutions, it is impossible to measure its activity. It was, however, found that a partial hydrolysate of DNP-hC-I was not only soluble in aqueous solution, but also possessed a strong tuberculin activity. This finding, though indirect and qualitatively, seems to indicate that the unhydrolysed DNP-hC-I is also active as tuberculin.

Thirty mg. of DNP-hC-I was suspended in 10 ml. of concentrated

Table II

Tuberculin Activity of hC and Its Derivatives

Preparation of tuberculin protein	Does (μg.)	Number tested	Average dimension of reaction (mm.)	
			Preparation of tuberculin protein	O. T.
hC	0.05	19	17×21	16×20
DNP-hC-S	0.05	9	11×11	11×12
PAB-hC	0.05	14	17×20	17×20

hydrochloric acid and allowed to stand at 37°. DNP-hC-I became solubilized after about one hour's standing. At various time intervals, 0.1 ml. of the solution was pipetted out, neutralized with 5 *N* NaOH, diluted with physiological saline solution to a concentration corresponding to 4 μg. of original

Table III

Tuberculin Activity of Hydrolysate of DPN-hC-I

Duration of hydrolysis (hrs)	Dose (μg.)	No. of tested	Average dimension of reaction (mm.)	
			Hydrolysate	O. T.
0[1)]	0.4	2	3×3	12×13
2	0.4	2	13×13	16×16
6	0.4	2	12×13	16×22
24	0.4	2	3×4	24×16

1) The test at 0 time was confirmed for the supernatant solution after centrifugation.

protein per ml., and used for the measurement of tuberculin activity.

As may be seen from Table III, soluble tuberculin-active peptides seem to appear in rather earlier stage of the reaction. They were, however, almost inactivated when the reaction was continued for 24 hours.

N-Terminal Amino Acid of hC—Fifty mg. portions of DNP-hC-I were hydrolyzed with 5 ml. of 6 *N* HCl in a sealed tube by heating at 110° for 5, 8 and 12 hours. The hydrolysates thus obtained were diluted with 30 ml. of water and extracted with ether, but no DNP-amino acid could be extracted into ether. The aqueous phase of the hydrolysate was dried *in vacuo* and subjected to paper chromatography according to the method of Koch and Weidel (*18*). No spots due to DNP-arginine and DNP-histidine could, however, be detected by this method. It seems therefore either that the protein has no N-terminal amino acid or that the N-terminal residue is so special that its detection by the DNP-method is very difficult.

p-Phenylazobenzoylation of hC with PABC—Fifteen ml. of acetone containing 50 mg. PABC were added to 100 mg. of hC dissolved in 85 ml. of 0.05 *M* Na_2HPO_4. After the solution was allowed to stand for 24 hours at room temperature, the reaction product (PAB-hC) was precipitated from the mixture by acidification to pH 3 with 2 *N* HCl. The precipitate was washed with acetone until the washing became colourless and dissolved in 40 ml. of dilute alkaline solution. After removing a small amount of insoluble material by filtration, the solution was again acidified to pH 3, and the precipitate formed was collected by centrifugation and washed with alcohol and ether. PAB-hC thus obtained was orange powder.

PAB-hC is soluble in neutral and alkaline solution, insoluble in acid solution. The absorption spectrum of PAB-hC in 0.1 *N* NaOH is shown in Fig. 1. The spectrum has absorption peaks at 325 mμ and 430 mμ.

Determination of PAB-Residues in PAB-hC—The number of PAB-residues introduced in hC was determined spectrophotometrically at 340 mμ or 330 mμ (*19*). The amount of original hC present in PAB-hC, on the other hand, was determined by estimation of amide nitrogen. From these data it was calculated that 27 PAB-residues was introduced into 10^5 g. of hC.

Tuberculin Activity of PAB-hC—As seen in Table II, PAB-hC was as potent as unmodified hC indicating that the phenylazobenzoylation had no effect on the tuberculin activity.

DISCUSSION

There are several reports concerning the relation between the chemical structure of tuberculin proteins and their biological activity. Eberson (*20*) acylated the crude tuberculin with acetic acid without appreciable loss of the activity. Ishida (*10*) reported that the treatment with nitrous acid, formaldehyde and other reagents which are known to react with the amino group failed to significantly affect the biological activity of tuberculin protein. In contrast to these results, Takeya and Mifuchi (*9*) found a correlation between the loss of tuberculin activity and the loss of free amino group as measured by Van Slyke's method when the tuberculin protein was treated with formaldehyde or nitrous acid, although only partial inactivation was attained by these treatments.

In the present investigation the effect of dinitrophenylation and phenylazobenzoylation of the tuberculin protein, hC, on its tuberculin activity was followed in relation to the number of modifying reagents introduced.

As can be seen from Table I, 9 out of the total 25 lysine residues and 12 out of the total 14 tyrosine residues in 10^5 g. of hC were dinitrophenylated under the condition employed. It was also demonstrated that the tuberculin activity of the modified protein remained unaffected as can be seen from Table II. Moreover, 15 out of the total 25 lysine residues and all tyrosine residues were dinitrophenylated under the conditions described by Sanger and it was indirectly shown that the tuberculin activity was still retained even after such a rather drastic treatment. It was also demonstrated that

the tuberculin activity was not influenced even after the 27 residues of PAB-radical were introduced into 10^5 g. of hC, which possesses 39 residues susceptible to phenylazobenzoylation. It seems, therefore, very likely that the lysine residues of hC, at least those half of total residues, and tyrosine residues are not directly concerned in the activity.

It is interesting that only half of the lysine residues are dinitrophenylated by the Sanger's DNP-method as can be seen in Table I. The reactivity of ε-amino groups of the lysine residues toward DNFB generally depends on the denaturing states of the proteins. hC is, however, a protein which has been sufficiently heated during preparation and its tyrosine residues are quantitatively dinitrophenylated under the same conditions. The half of the lysine residues in hC is, therefore, thought to be masked chemically by intramolecular linkages.

Kasuya and coworkers (*21–23*) found β-alanine as N-terminal residue of tuberculin active peptides and proteins obtained from the culture filtrate or bacterial cells of *human strain Aoyama B* using the DNP-technique. In contrast, Yamamura and coworkers found no N-terminal amino acid* in a crystalline tuberculin-active peptide (*24*) obtained from the bacterial cells by means of the DNP-method. No N-terminal residue could also be detected in hC in the present investigation by means of the usual DNP-method. This fact seems to suggest that this protein is a cyclic peptide chain or that the N-terminal residue is so special that its detection by the DNP-method is very difficult.

SUMMARY

1. Tuberculin-active protein, hC, was dinitrophenylated with DNFB, but the tuberculin activity was not affected even after half of the lysine residues and all of the tyrosine residues were blocked by this reagent.

2. When PABC was reacted with hC, 27 PAB-residues was introduced into 10^5 g. of protein. Tuberculin activity remained unchanged after this treatment.

3. No N-terminal amino acid was found in hC by means of the DNP-method.

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FORMATION OF SERUM γ-GLOBULIN IN LIVER*

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The *in vitro* biosynthesis of proteins has been extensively studied by many investigators, largely by means of the incorporation of a labeled amino acid into proteins; much important and attractive evidence has been reported in terms of amino acid activation, energy requirement, relationship with nucleic acids *etc.*, which might be deeply concerned with the mechanisms involved in protein synthesizing reactions. Nevertheless, such a rather indirect method, although affording a great advantage for research in this field, leaves open the question of whether or not a protein or peptide bond is actually synthesized.

On the other hand, approaches for the direct demonstration of protein synthesis have been considerably few in number because of the difficulties in detecting small quantities of certain proteins selectively. But with respect to proteins having biological activity, such as some kinds of enzymes and antibodies, there appeared several reports utilizing their specific activities as a clue of determination.

By making use of the serological quantitative precipitin reaction, which was proposed by Heidelberger *et al.* (*3–5*) as a method useful to overcome such difficulties, Peters and Anfinsen (*6*) observed a net production of serum albumin by chicken liver slices. They demonstrated that 0.12 mg. of this protein was produced in one hour per g. of wet weight of liver and further, the existence of its precursor was suggested (*7*). Campbell and Stone (*8*), using a similar method, investigated the net synthesis of albumin by liver slices of normal and tumor-bearing rats, obtaining the results that the latter case showed a diminished activity.

Following these researches, the authors have made a study on the net production of serum γ-globulin in horse liver, although isotopic studies of other investigators dealing with the formation of γ-globulin as well as antibody globulins by liver preparations have shown only a minor activity of this tissue compared with other lymphatic organs.

The present report describes the results of this study using liver slices and cell free preparations, including a soluble system.

* A greater part of this work has been already published in Japanese (*1*, *2*).

EXPERIMENTAL

A. Quantitative Precipitin Reaction

Antigen—Horse serum γ-globulin was purified by combination of the ethanol fractionation method according to Cohn *et al.* (*9*, *10*) with the salting out procedure employing sodium sulfate. The obtained preparation was found to be almost pure in the analysis by electrophoresis using a Tiselius apparatus as well as by the solubility test in a solution of a neutral salt.

Preparation of Antiserum—Normal rabbits were injected intravenously with an increasing amount of a sterile solution of the purified γ-globulin (0.25 per cent) three times a week for a period of 5 to 6 weeks. Total amount of the injected γ-globulin was 80 to 100 mg. per one rabbit. One week after the last innoculation rabbits were sacrificed by bleeding, and sera were separated and pooled to be lyophilized. Prior to use the powder was dissolved in distilled water to make its protein concentration 2 per cent and centrifuged at 3,000 r.p.m. for 20 minutes to remove insoluble materials. This antiserum can retain its activity for a fairly long period of time when stored in a desiccator in the state of dried powder.

As for its specificity, the antiserum thus obtained did not cause a cross reaction with any component of serum proteins other than γ-globulin so far as examined by immunoelectrophoresis (*11*).

Procedure—According to the method of Heidelberger *et al.*, a sample to be evaluated for its γ-globulin content was mixed with a certain quantity of antiserum in a centrifuge tube, which was kept standing at 37° for 2 hours and then in a refrigerator for 15 to 20 hours. The specific precipitate thus formed was separated by centrifugation in the cold, washed twice with a small amount of ice-cold water by repeated centrifugation, dissolved in a dilute alkali solution, and its protein content was determined colorimetrically by the use of Folin's phenol reagent (*12*). The sample was previously centrifuged at $14{,}000 \times g$ for 15 minutes, if necessary, to minimize a non-specific precipitation.

In all cases duplicate samples were taken and two kinds of blank consisting of antiserum or sample alone were run simultaneously. The mean of the obtained values for duplicate samples was corrected by subtracting the values of the blanks. The details of this procedure have been described in a previous paper (*1*).

B. Experiment Using Liver Slice

Liver Slice—Normal horse liver was sliced with a hand slicer in the thickness of less than 0.5 mm. In order to eliminate blood components, slice was washed repeatedly with a sufficient volume of ice-cold medium, blotted onto a filter paper and weighed by a torsion balance.

Medium—Unless indicated, a salt mixture proposed by Peters and Anfinsen (*6*) was used as a medium, of which composition was as follows: (millimoles per liter)

Medium A;	NaCl	105,	$NaHCO_3$	30,
	$KHCO_3$	10,	$CaCl_2$	10.

Determination of Net Production—About 500 mg. of slice, exactly weighed, was suspended in 15 ml. of medium and incubated at 37° for 2 hours under a gas phase of air. After incubation the amounts of γ-globulin contained in slice and medium were determined separately by means of the quantitative precipitin reaction above described. The slice was homogenized with a glass homogenizer under cooling, centrifuged at $14{,}000 \times g$ for

15 minutes and the resulting supernatant was submitted to determination. The net production was calculated as a difference between the sum of these two values and the amount of γ-globulin which was present in the slice before incubation.

Hydrolysate of Plasma Proteins—Human plasma proteins were hydrolyzed by boiling with 5 *N* sulfuric acid for 35 to 40 hours. After neutralization and removing sulfate by the addition of barium hydroxide, the hydrolysate was adjusted to a volume the same as the original plasma and tryptophan was added to a final concentration of 50 mg./dl.

C. *Experiment Using Liver Homogenate*

Liver Homogenate—Normal horse liver was first sliced as above, weighed and homogenized with a glass homogenizer using three times the volume of cold medium.

Cell Fractionation—According to the method of Zamecnik *et al.* (*13*) the cell fractionation was carried out by centrifugation with use of a Spinco Model E Ultracentrifuge, a Hitachi Preparative Ultracentrifuge and an International Refrigerated Centrifuge PR-1.

Medium—Besides Medium A, the same solution as Medium X designed by Zamecnik *et al.* (*13*) was preferably used, of which composition was given as follows: (millimoles per liter)

Medium X;	sucrose	350,	$MgCl_2$	10,
	$KHCO_3$	35,	KCl	25,
	potassium phosphate buffer (pH 7.4)			20.

Determination of Net Production—The conditions for incubation of the homogenate were the same as those in the case of the slice, and an increase in γ-globulin content during incubation corresponded to the net production.

Adenosine Triphosphate (*ATP*)—ATP was prepared from rabbit muscle as a barium salt by the method of Kerr (*14*) and Dounce *et al.* (*15*). The purification procedure was repeated three times. Just prior to use, it was transformed to a sodium salt and added to the system at a final concentration of 3.5×10^{-3} *M*.

D. *Experiment with Deoxycholate Soluble System*

Deoxycholate Soluble System—Liver homogenate prepared as above was centrifuged at $700 \times g$ for 10 minutes, or $5{,}000 \times g$ for 10 minutes in later experiments, to remove cell debris, the nuclear fraction and, in the latter case, the mitochondria as well. The resulting supernatant was centrifuged again at $22{,}000 \times g$ for 60 minutes or $105{,}000 \times g$ for 30 minutes to separate the remaining particulate fractions from the supernatant.

The latter supernatant was deproteinized by the addition of trichloroacetic acid (TCA; final 5 *g.*/dl.) or perchloric acid (PCA; final 0.6 *M*) and neutralized with a solution of sodium or potassium hydroxide, making its final volume to one and a half of the original supernatant. On the other hand, the precipitated fractions were suspended in a cold tris-hydroxyaminomethane buffer containing 0.5 or 1.0 per cent sodium deoxycholate and kept standing overnight in a refrigerator to complete the solubilization of the proteins involved in those particles. And then they were centrifuged again at $22{,}000 \times g$ for 60 minutes or $105{,}000 \times g$ for 30 minutes to obtain clear supernatant. All these procedures were carried out at a low temperature, around 0°.

The two obtained preparations were combined again to be incubated after fortification with ATP for the observation of the net production of γ-globulin.

pH 5 Enzyme—By the method of Keller *et al.* (*16*) using 1 *N* acetic acid, a pH 5 enzyme was prepared from the supernatant fraction of horse liver.

Ribonuclease (*RNase*)—Crystalline pancreatic RNase was purchased from the Sigma

Company.

Guanosine Triphosphate (GTP)—A sodium salt of GTP obtained from the Pabst Laboratory was used at a final concentration of 2.5×10^{-4} *M*.

Estimation of RNase Inhibition—By a modification of Roth's method (*17*), yeast ribonucleic acid (RNA) was incubated with RNase for a definite period of time at room temperature in the presence and absence of the fractions to be estimated, and then the remaining RNA was determined according to Mejbaum (*18*). An inhibiting activity was expressed by a percentage decrease in the hydrolyzed RNA.

RESULTS

A. Calibration Curve for Precipitin Reaction

Using a known and varying quantity of the purified serum γ-globulin, the calibration curve as shown in Fig. 1 was established. A linear relationship was demonstrated in the range of 0 to 50 μg. of γ-globulin. It was also recognized that the amount of each precipitate (antigen-antibody complex) was 5 times greater than that of γ-globulin (antigen) alone.

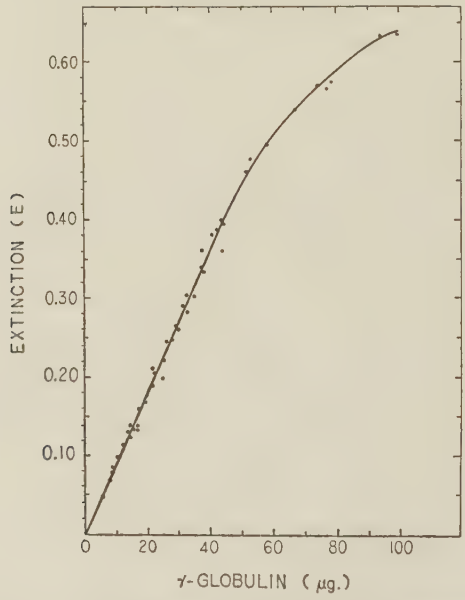

FIG. 1. The calibration curve for the determination of horse serum γ-globulin by the quantitative precipitin reaction.

B. Net Production of γ-Globulin by Liver Slice

Net Production—When a liver slice was incubated with Medium A under the above conditions, a net production of γ-globulin was observed to the extent of 136 ± 7.6 μg. in 2 hours per g. of wet weight of liver. This value

has been calculated from the results of 20 experiments, of which representatives are listed in Table I.

Net Production in Various Media –This net production was enhanced by using acid hydrolysate of plasma proteins as a medium and markedly reduc-

TABLE I

Net Production of γ-Globulin by Liver Slice

γ-Globulin before incubation	γ-Globulin after incubation			Net production
	Medium	Slice	Total	
530	590	90	680	150
565	615	50	665	100
530	595	65	660	130
610	655	95	750	140
575	620	90	710	135

Liver slice was incubated at 37° for 2 hours with Medium A under a gas phase of air. Values were expressed as μg. of γ-globulin per wet weight of liver.

TABLE II

Net Production of γ-Globulin with Various Media

Media		Net pruduction (%)
Medium A (control)		100
Krebs-Ringer bicarbonate buffer		95
,, phosphate buffer		100
Plasma protein hydrolysate		167
Medium A+monoiodoacetate	$10^{-2}M$	0
	10^{-3} ,,	28
	10^{-4} ,,	100
,, +2,4-dinitrophenol	10^{-3} ,,	0
	10^{-4} ,,	55
,, +NaF	10^{-2} ,,	15
,, +Arsenite	10^{-3} ,,	45
,, +Arsenate	10^{-3} ,,	62

Net production was exhibited as per cent, taking the control run as 100. All conditions were the same as those in Table I.

ed by adding several metabolic inhibitors such as dinitrophenol, monoiodoacetate *etc.* to the basal medium. But two kinds of Krebs-Ringer's buffer were found to make no difference with Medium A, so far as examined. These results are shown in Table II.

C. Net Production of γ-Globulin in Liver Homogenate

Net Production and Effect of ATP—Liver homogenate was observed to show a remarkable loss in the activity of producing γ-globulin compared with the liver slice, but it was recovered by nearly 70 per cent by fortification with ATP at a concentration of $3.5\times10^{-3}\,M$ as indicated in Table III.

TABLE III

Net Production of γ-Globulin by Liver Homogenate

Preparation	Medium	ATP (*M*)	Net production (%)
Slice	Medium A	0	100
Homogenate	,,	0	15
Homogenate	Medium A	7×10^{-4}	32
		1.8×10^{-3}	38
		3.5×10^{-3}	68
Homogenate	Plasma protein hydrolysate	0	25
		3.5×10^{-3}	78

From the finding that plasma protein hydrolysate was far less effective than ATP, it would be supposed that a limiting factor for the formation of γ-globulin in homogenate might be mainly ATP as a source of energy or a co-factor rather than amino acids as materials for protein synthesis.

Gas Phase - Employing a Warburg apparatus, gas phase under which homogenate was incubated with ATP was investigated. Taking the net production under air as 100, that of 127 and 47 per cent were observed under oxygen and nitrogen respectively.

Activity in Various Cell Fractions - In order to study the site of γ-globulin formation, various cell fractions prepared by a differential centrifugation were estimated for their activities to produce this protein in the presence of additional ATP.

From the results as shown in Table IV, the following evidence should be noticed.

a) The supernatant fraction separated by centrifugation at $5{,}000\times g$ for 10 minutes, retains 70 per cent of the activity of whole homogenate.

b) Net γ-globulin production is not demonstrated by the particulate fractions alone, suggesting that the soluble components are required for the full activity.

c) There remains a considerable activity in the supernatant fraction after centrifugation at $105{,}000\times g$ for 30 minutes.

Deproteinization of Supernatant—The supernatant fraction separated by centrifugation at $105{,}000\times g$ for 30 minutes was treated with TCA (final 5 g./dl.), followed by neutralization with sodium hydroxide. This deproteinized supernatant, when combined again with the particulate fractions and incubat-

ed with ATP, gave the almost same amount of γ-globulin production as the non-treated supernatant. Because of a possibility that the treatment with TCA might remove a factor causing decomposition of γ-globulin preferably

TABLE IV

γ-Globulin Producing Activity of Subcellular Fractions

Cellular fraction	Net production	
Whole homogenate	145 (μg.)	100 (%)
Supernatant at 700×*g*, for 10 mins.	130	89.5
,, at 5,000×*g*, for 10 mins.	100	69
,, at 105,000×*g*, for 30 mins.	50	38
All particulate fractions[1)]	0	0

Incubation was carried out with addition of ATP at a final concentration of 3.5×10^{-3} *M*. Net production was expressed as μg. of γ-globulin per g. of wet weight of liver. Medium X was used.

1) The precipitates separated by centrifugation of the whole homogenate at 105,000×*g* for 30 minutes.

to its net production, one can not come to the conclusion that the deproteinized supernatant contains all effective factors contained in the original supernatant. But for the sake of the convenience of eliminating γ-globulin which existed prior to incubation, further experiments were carried out with use of this deproteinized supernatant.

D. Net Production in Deoxycholate Soluble System

Net Production—In order to obtain a soluble system for protein formation which would practically facilitate further study, both particulate and supernatant fractions were treated separately. As described in the section of Experimental, the former was treated with deoxycholate by which involved proteins were brought into solution, and the latter was deproteinized on the basis of the above result.

These two preparations, *i.e.* deoxycholate soluble proteins of the particulate fractions and TCA or PCA soluble components of the supernatant were combined again to be incubated with ATP. Even in such a soluble system the net production of γ-globulin could be clearly demonstrated (Table V).

Dialysis of the deproteinized supernatant resulted in a complete loss of its activity. Similar fractions derived from other animals were able to replace the horse supernatant. RNase was found to show only a partial inhibition to this system.

Effect of Plasma Protein Hydrolysate and pH 5 Enzyme—Systems using instead of the deproteinized supernatant, plasma protein hydrolysate and/or a pH 5 enzyme preparation, were examined. As demonstrated in Table V, the hydrolysate of plasma proteins could not replace completely the superna-

tant fraction even together pH 5 enzyme, indicating that in the supernatant there might be some effective factors other than amino acids. The pH 5 enzyme did not exhibit an additive effect when used with the deproteinized supernatant so far investigated.

TABLE V

Net Production in Deoxycholate Soluble System

Supernatant fraction	γ-Globulin produced (μg.)				
	I	II	III	IV	V
Deproteinized supernatant	45	50	45	60	65
,, ,, + pH 5 enzyme				60	
,, ,, + RNase[1)]					50
Plasma protein hydrolysate			20		
,, ,, ,, + pH 5 enzyme	30	45	35		
pH 5 enzyme	10				
Deproteinized supernatant from rat					100

I-V indicate experimental numbers. Values are based on 1 g. of wet weight of liver. The incubated system consisted of: extract of the particulate fractions with deoxycholate; each fraction as indicated above; ATP 3.5×10^{-3} *M*.

1) RNase was added at a final concentration of 0.05 mg./ml.

Effect of ATP and GTP—Table VI shows the effect of ATP and GTP on this soluble system. These nucleotides have been reported to be necessary for protein synthesis in terms of the activation of amino acids and their incorporation into microsomal proteins (*16*, *19*, *20*). It is interesting that GTP has been found more efficient than ATP.

TABLE VI

Effect of ATP and GTP

Nucleotide	γ-Globulin produced (μg.)			
	I	II	III	IV
ATP	40	60	65	35
GTP	60	85		45
ATP and GTP		90	75	

Experiments were carried out in the deoxycholate soluble system.
ATP: 3.5×10^{-3} *M*, GTP: 2.5×10^{-4} *M*.

Fractionation of Solubilized Proteins—By treatment with 1.0 per cent sodium deoxycholate, 80 per cent of microsomal proteins and 40 per cent of its RNA came into solution. The ratio of RNA to protein in the microsome, deoxycholate soluble and insoluble fractions were 0.062, 0.03 and 0.193 respectively.

Extracted proteins of microsomes were subjected to fractionation by salting out with ammonium sulfate. As it is known from Table VII, almost all the active protein components would be contained in a fraction precipitable between the concentration of 10 to 20 g./dl. of this salt solution, which occupies 20 per cent of the total protein in quantity. But it should be mentioned that some non-protein factors are responsible for the whole activity of the extract, which has been fully recovered by the addition of them to the fractionated proteins.

Table VII

Protein Fractionation and Activity

Fraction	γ-Globulin produced (μg.)[1]	Protein fractionated (%)[2]
Whole extract	50	100
Precipitate with 0–10 g./dl. $(NH_4)_2SO_4$	0	78
,, ,, 0–20 ,, ,,	27.5	96
,, ,, 0–30 ,, ,,	30	97

The incubated system consisted of: each fraction as indicated above; the deproteinized supernatant; ATP 3.5×10^{-3} *M*.

1) Values were expressed on the basis of g. of wet weight of liver.

2) Protein concentrations were determined by the biuret reaction.

RNase Inhibition by Deproteinized Supernatant—Active factors involved in the deproteinized supernatant have been investigated by column chromatography with use of ion exchange resins. They were not lost by passing through a column of Dowex-1 formate, but were adsorbed by a Dowex-50 resin (H-form).

Considering the lesser effect of RNase on this protein synthesizing system, there might be a possibility that RNase inhibitors exist in the supernatant fraction and make a contribution to the protein synthesis through a stabilizing action for ribonucleoproteins. The data indicated in Table VIII may support this possibility.

Besides this fact, the components adsorbed onto a column of Dowex-50 have been fractionally eluted with varying concentrations of hydrochloric acid and each eluted fraction has been studied for γ-globulin producing activity as well as its RNase inhibiting action. The results so far obtained show a good correspondence between these two activities, suggesting that RNase inhibitors play a significant role for γ-globulin formation among the effective factors in the deproteinized supernatant.

TABLE VIII

Treatment of Deproteinized Supernatant with Ion Exchange Resin

Fraction	γ-Globulin produced ($\mu g.$)[1]	RNase inhibition (%)[2]
Untreated deproteinized supernatant	55 (20)	40
Dowex-1 non-adsorbed fraction	60 (0)	25
Dowex-1 adsorbed fraction	0	—
Dowex-50 non-adsorbed fraction	0	0

The incubated system consisted of; the whole extract of particulate fractions with deoxycholate; each treated fraction as indicated above; ATP 3.5×10^{-3} *M*.

1) Values were expressed as before. Those in parenthesis showed the results in the absence of ATP.

2) See Experimental. 1 ml. of each examined fraction or distilled water 1 ml. of RNase solution (0.1 μg./ml.), 1 ml. of acetate-veronal buffer (0.1 *M*, pH 7.8) and 1 ml. of RNA solution (2 mg./ml.), were mixed and incubated for 20 minutes at room temperature.

DISCUSSION

There have been considerably few reports concerning the synthesis of serum γ-globulin in liver among which those of Miller *et al.* (*21*) and of Asconas *et al.* (*22*) have inclined to deny that possibility. But the present work which has demonstrated clearly the net production of γ-globulin by liver slices and further, a cell free system of liver, has supported the idea that liver might have an ability to produce serum γ-globulin. Employing a similar method, Hirayama *et al.* (*23*, *24*) also observed a net production of serum γ-globulin by normal and injured liver slices from human beings and rats, and reported a measurable incorporation of a labeled amino acid into this protein under the same experimental conditions. From the value obtained in the case of slices, the metabolic rate of serum γ-globulin was approximately estimated (*1*). The calculated half life of around 60 days was considerably greater than that from turnover experiments *in vivo*, suggesting a extra-hepatic formation of this protein.

Nevertheless, the net production observed in the present investigation is not merely a release of γ-globulin from particles into a medium, but involves some steps of biosynthesis, which is supported by the following evidence.

a) In slices, the increase in net production obtained by using plasma protein hydrolysate as a medium and contrary, the remarkable decrease obtained by adding various metabolic inhibitors into a medium.

b) The pronounced effect of ATP in the cell free system.

c) The failure to detect net production by incubation of particulate fractions alone even in the presence of additional ATP.

d) Findings in the deoxycholate soluble system.

Many investigations so far published have favored the microsomes and the supernatant fraction as the principal site of protein synthesis. Although the present results are consistent with those findings, it is difficult to estimate definitely what role these fractions really play in the formation of γ-globulin within a living body, because the differentiation of the subcellular components by centrifugation only is so incomplete that any obtained fraction might be contaminated with other components, and also the requirement of any fraction to be fortified with ATP for the observation of net production makes the explanation complicated.

Apparently some differences can be noticed between the present results and those from other experiments dealing with the labeling of proteins with various amino acids. These include: a) The necessity for the pH 5 enzyme which has been reported to be essential for amino acid activation (*20*), is not distinct in the authors' system. b) The supernatant fraction after centrifugation at $105{,}000 \times g$ retains a considerable activity for γ-globulin net production. c) Ribonucleoprotein particles (*25*) playing a determinant role in the amino acid incorporation have been found to remain rather insoluble in sodium deoxycholate, but the authors have used the deoxycholate soluble fraction. d) In labeling experiments, non-protein components in the supernatant fraction have been considered not so important as in the present system. e) RNase has shown only a partial inhibition in this case, while it has suppressed the amino acid incorporation absolutely.

These discrepanicies might be generally understood by a probable contamination of active factors with each other because of the differences in the experimental conditions. But an alternative possibility should be taken into consideration; that the incorporation of a single amino acid in a short period of time and the net production of a whole protein molecule would reflect somewhat different steps involved in a complex process of protein synthesis. Actually, the pattern of labeling after incubation for a longer time has shown a marked change compared to that usually obtained in a short period of time. Also there have appeared recently some papers reporting an RNA-less protein fraction of microsomes being labeled most rapidly, and others reporting active factors of the supernatant fraction which are heat-stable and dialysable.

A stabilizing factor for the ribonucleoprotein particles contained in a TCA or PCA soluble supernatant fraction of liver has been indicated (*26*, *27*). The authors' study has really suggested a close relationship between such an RNase inhibitor and the protein formation. It should be regarded as one of the most important factors in the supernatant, and is now under investigation.

As a cell free system for protein synthesis in terms of net production, Straub *et al.* (*28*) reported the system for the formation of amylase by pigeon pancreas, using its water extracts, casein hydrolysates, L-ascorbic acid and a larger amount of ATP. The deoxycholate soluble system of the present authors also gives a great convenience for further study, but it remains to be decided by future research whether this system which supports

only 50 per cent of the total quantity of γ-globulin net production would cover qualitatively all of the reactions involved in the formation of this protein.

SUMMARY

A study to investigate the ability of liver to synthesize serum γ-globulin has been made by the detection of its net production with an application of the quantitative precipitin reaction. The following results have been obtained:

1. The liver slice, when incubated in a medium of salt mixture, could produce 136 ± 7.6 μg. of γ-globulin in 2 hours per g. of wet weight of liver. This net production was enhanced by using acid hydrolysates of plasma proteins as a medium and markedly reduced by adding several metabolic inhibitors to the medium.

2. The homogenate could not reproduce this activity under the same condition as in the case of the slice, but it was almost recovered by fortification with ATP at a final concentration of 3.5×10^{-3} M.

3. With use of various subcellular fractions prepared by differential centrifugation, it was demonstrated that the microsomes, in combination of the supernatant fraction, retained about 70 per cent of the activity compared to the whole homogenate, and the particulate fractions alone showed no detectable net production.

4. In the deoxycholate soluble system which consisted of the deoxycholate soluble components derived from the particulate fractions, TCA or PCA soluble factors of the supernatant fraction and ATP, authors have demonstrated the net production of γ-globulin, though to a limited extent.

5. In this soluble system, active proteins in deoxycholate extracts were able to be salted out with between 10 and 20 g./dl. of ammonium sulfate solution, and RNase inhibitors, although unknown, were indicated as one of the important factors involved in the deproteinized supernatant. Compared to ATP, GTP was found more efficient.

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STUDIES ON INSULIN

I. TWO DIFFERENT INSULINS FROM LANGELHANS ISLET OF BONITO FISH*

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From Langelhans islet of fish, an appreciable amount of insulin has been prepared for clinical purposes, in Japan.** The islet, differing from those in mammalian pancreas, is present as an isolated gland mainly composed of β-cells in which the hormone is synthetized and reserved. Thus partially purified insulin of 15 to 19 I.U. per mg. has been easily obtained in an excellent yield by extracting the isolated gland, followed by the fractionations with acetone, with sodium chloride, then at the isoelectric point. Further purification of fish insulin, however, has been accomplished with much more difficulty and with much more loss of activity, than purification of mammalian insulin.

As already reported by Honma and Hiraoka (*1*), both insulins from fish and from mammal though having just the same hormonal and antigenic*** characters, have been found to possess some physical properties different from each other. As to the chemical structure of both insulins, too, there have been reported marked differences; Akabori and Ohno (*3*) found asparagine and prolyl-lysine as the C-terminals of bonito insulin, instead of asparagine and prolyl-lysyl-alanine in mammalian ones (*4*, *5*); while Nagasawa and Nishizaki (*6*) reported the presence of three different N-terminals in bonito insulin, glycine, alanine and leucine, instead of two different N-terminals, glycine and phenyalanine, in mammalian ones (*4*, *5*)

In this paper we wish to report that partially purified insulin from Langelhans islet of bonito, could be separated on a column of CM-cellulose into two components having full insulin-activity; and that the one had one glycyl and one leucyl chains, while the other had one glycyl and one alanyl chains per 6×10^3 g., respectively. In addition, it will be described some evidences that the leucyl and alanyl chains in bonito insulins corresponded to the phenylalanyl chains in mammalian ones, and some differences observed between

* The abbreviation used in this paper, are I.U. for the International Unit of insulin; CM for carboxymethyl; DNP for 2,4-dinitrophenyl.

** The annual production in 1958, was estimated to be more than 10^7 I.U.

*** Yamamura, Y., Personal communication. See also (*2*).

the properties of both insulins from fish and from mammal, seemed to be mainly due to the structural difference of the both glycyl chains.

EXPERIMENTALS

Materials—Langelhans islets of bonito fish, which had been freshly isolated and stored in one per cent picric acid solution at 0 to 5°, were kindly offered from Shimizu Seiyaku Co. Ltd., Shimizu. Partially purified bonito insulin, and crystalline beef and whale insulins were also supplied from the company.

CM-Cellulose used for chromatography, was prepared in this laboratory from cellulose powder ('for chromatography', Toyo Roshi Co. Ltd., Tokyo) according to the method of Peterson and Sober (*7*).

Methods—Chromatography of insulins was achieved at room temperature on a column of CM-cellulose which had been equilibrated previously by washing with *N*/2 sodium acetate at pH 4.0. For analytical purposes, we used columns of 1.5×13.5 cm., with 1.8 g. of CM-cellulose (air dried weight), while much larger ones (3×28.5 cm.) for preparative purposes. As soon as the applied insulin solution at a concentration of ten per cent in *N*/2 acetic acid (about one-fiftieth volume of the column used) had been allowed to flow into the column, a continuous inflow of a mixture of *N*/2 acetic and *N* formic acids (4 : 1, v/v) was initiated. The latter mixture thus introduced was contained in a mixing vessel which, in turn, was supplied with *N* formic acid to maintain the mixture to be a fixed volume (ten-times volume of the column used). The rate flow was 5 ml. per hour per cm^2. (cross-section of column). The gradient change of the concentration of formic acid in mixing vessel, was calculated according to the following formula:

$$C = C_2 - (C_2 - C_1)\, e^{-v/V}$$

where C_1 was th initial concentration (*N*/5), C_2 was the concentration of supplier (*N*), C was the concentration in mixing vessel after the inflow of v ml., and V was the fixed volume of the mixture in ml. (*8*).

Protein content in solution was determined by direct spectrophotometry at 275 mμ and by turbidimetry in *M* trichloroacetic acid according to the method of Rasmussen and Satake (*9*).

N-Terminals were analysed by the usual DNP-method (*9*, *10*). The DNP-amino acids in acid hydrolysates (6 *N* HCl, 110°, for 5 hours) were identified by paper chromatographically on Toyo Roshi No. 51 A, with the use of *n*-butanol saturated with 5 per cent ammonia and 1.5 *M* sodium phosphate buffer at pH 6.0 as the two solvent systems. For quantitative determination the yellow spots were eluted separately with 4 per cent sodium bicarbonate, and the optical density at 360 mμ was determined for each extract. The over-all recovery of DNP-amino acid was assumed to be 95 per cent for ε-mono-DNP-lysine, to be 75 per cent for DNP-alanine and -leucine, to be 65 per cent for N_1-mono-DNP-histidne (as 2,4-dinitroaniline), and to be 40 per cent for DNP-glycine, respectively (*9*, *10*).

The oxidative cleavage of disulifide linkages of insulins was accomplished with performic acid, according to the method of Sanger (*11*). The oxidized insulin solution so obtained was directly separated into A and B chains by the following methods; electrophoresis on paper (Toyo Roshi No. 50) at a potential gradient of 8 volt per cm., using *N* formic acid (pH 1.9) as the medium (*5*); fractional precipitation with *M* trichloroacetic acid according to the method of Satake *et al.* (*12*); counter current distribution between the two layers (10 ml. each for transfering (upper) and stationary

(lower) phases) resulted from an equivolume mixture of 2-butanol and 0.077 *M* *p*-toluene sulfonic acid (*13*), with the use of completely automatic 'Craig type' apparatus (No. 555 F, Mitamura Shoten Co., Ltd., Tokyo). Partition coefficient (*K*) of the distributed solute was calculated from total transfer-number and tube-number having the solute at a maximum concentration. From the *K* value, theoretical distribution curve was calculated according to the method of Lieberman (*14*).

Bioassay of insulin was kindly done by the Bioassy Division of Shimizu Seiyaku Co., Ltd., according to the standard international method.

RESULTS

Bonito insulin used in this study, was prepared according to the methods as summarized in Scheme I. The partially purified preparation had an insulin-activity of 19 I.U. per mg., which corresponded to that of 80 per cent-

Scheme I

Preparation of Bonito Insulin

Langelhans islets
↓ extracted with acid acetone
75 per cent acetone-extract
↓ added with acetone
---- Crude bonit insulin ----
↓ dissolved into water
↓ added with NaCl
Precipitates between 15–25 per cent NaCl
↓ dissolved into *N*/100 HCl
↓ added with NaOH, in the presence of Zn^{++}
Precipitates at pH 5.2
----- Partially purified bonito insulin -----

pure beef insulin. By the use of DNP-method, however, the preparation was found to possess three different N-terminals, glycine, alanine and leucine* at a ratio of 0.95, 0.45 and 0.55 mole, together with 1.0 mole of lysine and 1.8 mole of histidine residues per 6×10^3 g.,** respectively. The results would seem to suggest that bonito insulin had a structure and an insulin-activity quite different from those of mammalian ones (*4*), if it were not for the fact that the bonito insulin preparation possess a heterogeneous character.

Fig. 1 shows the elution diagram of bonito insulin from a column of CM-cellulose by the use of concentration gradient of formic acid from *N*/5 up to *N*. Component corresponding to the first peak eluted after the hold-

* Differentiation between DNP-leucine and -isoleucine which could not be distinguished from each other by the use of the method described in this paper, will be described in succeeding paper.

** The accuracy was within ±0.05 mole per 6×10^3 g.

up volume, had some amounts of tryptophan residue and showed only a trace of insulin-activity. Both components corresponding to the second (bonito insulin I) and the third (bonito insulin II) peaks, on the other hand, possessed a high insulin-activity essentially the same as that of crystalline beef insulin. Under the same conditions, the lyophilisate of each peak fraction was eluted as a single peak at the same position as the original elution position shown in Fig. 1, respectively. Crystalline beef and whale insulins were also eluted

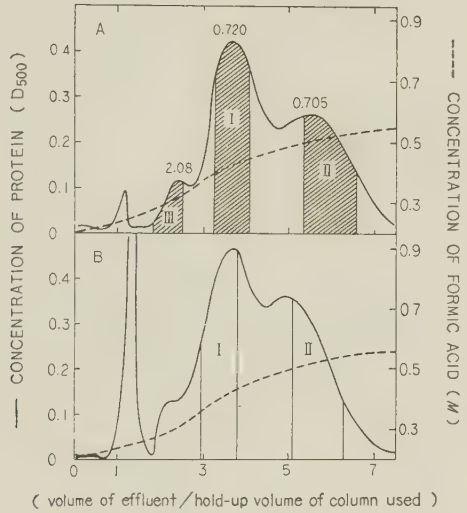

Fig. 1. Elution chromatography of partially purified (A) and crude (B) bonito insulins from CM-cellulose column.

Protein content in effluent was determined by the turbidity produced from 0.1 ml. of sample and 3 ml. of *M* trichloroacetic acid. The lyophilisate of peak fraction with shade, were used as the samples of bioassay, chemical analysis, and rechromatography. Each figures on the peak present the optical density of 0.1 per cent solution at 275 mμ.

as a single peak but at a different position between those of bonito insulins I and II. In all cases, the recovery of insulin from the column was almost quantitative. The results seemed to indicate the presence of two different insulins in Langelhans islet of bonito fish.

By the use of DNP-method it was found that bonito insulin I was composed of glycyl and leucyl chains, while insulin II had glycyl and alanyl chains, though the both equally had two N-terminals, (1.8~2.0 mole), two histidine (1.7~1.9 mole) and one lysine (0.9~1.0 mole) residues per 6×10^3 g., respectively. As to the solubility, too, there was marked difference between the both; thus bonito insulin II was fairly soluble in water at the isoelectric

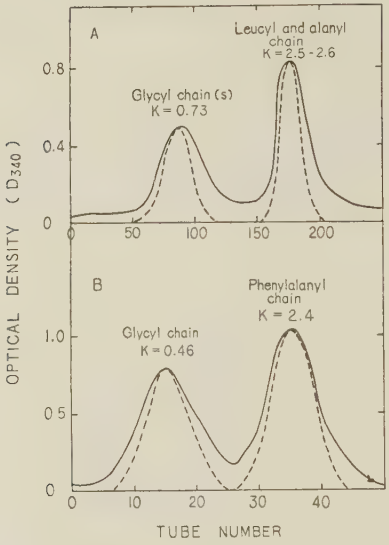

Fig. 2. Counter current distribution diagram of oxidized bonito (A) and beef (B) insulins.

Solvent system: Equivolume mixture of 2-butanol and 0.077 *M* *p*-toluene sulfonic acid.

Protein content was determined spectrophotometrically with 2,4,6-trinitrobenzene-1-sulfonic acid (*10*, *19*).

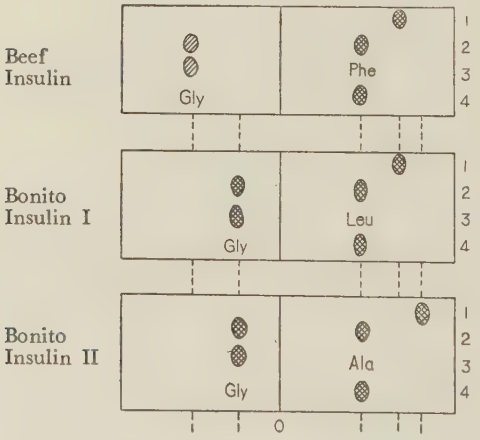

Fig. 3. Paper electrophoretic patterns of intact and oxidized insulins.

1) Intact insulin. 2) Performic acid-treated insulin; 3) *M* Trichloroacetic acid-soluble fraction derived from 2). 4) *M* Trichloroacetic acid-insoluble fraction derived from 2). ◍ Colored with ninhydrin; ◍ colored with Sakaguchi-reagent. Conditions are described in experimental part.

point,* especially in the absence of insulin I which precipitated almost completely at the isoelectric point just as mammalian insulin did.

Fig. 2A shows the counter current distribution diagram of chromatographically unfractionated bonito insulin, which had been previously treated with performic acid to cleavage the inter-peptide disulfide linkages. The N-terminal analysis of each peak fraction indicated that the slow moving peak fraction ($K=0.73$) corresponded to oxidized glycyl chains and the fast moving peak fraction ($K=2.5$–2.6) was a mixture of oxidized alanyl and leucyl chains, and that the lysine and the histidine residues located only in the fast moving fractions. In Fig. 2B is shown the distribution diagram of oxidized beef insulin between the same solvent systems.

The results might indicate that the leucyl and alanyl chains ($K=2.5$–2.6) in bonito insulins seemed to correspond to the basic phenylalanyl chain ($K=2.4$) in mammalian ones, and that the glycyl chain(s) in bonito insulins ($K=0.63$) seemed to be fairly different from that in mammalian ones ($K=0.46$).

The presumption was further varified by paper electrophoretic analysis of oxidized insulins. As shown in Fig. 3, phenylalanyl chains from beef insulin and leucyl or alanyl chains from bonito insulins were basic and showed just the same electromobility, while glycyl chain(s) from bonito insulins were much less acidic than that from beef insulin. The most remarkable fact was that the former glycyl chains, as well as the phenylalanyl, leucyl

TABLE I

Some Properties of Oxidized chains Derived from Bonito and Beef Insulins

	A-chain			B-chain		
	beef insulin	bonito insulin I	bonito insulin II	insulin	insulin I	insulin II
M Trichloroacetic acid (a)	sol.	sol.	sol.	insol.	insol.	insol.
Partition Coefficient (b)	0.46	0.73	0.73	2.4	2.5–6	2.5–6
Electromobility[1] (a) (cm^2/hour·volt)	−0.16	−0.06	−0.06	+0.14	+0.14	+0.14
N-Terminal (c)	Gly	Gly	Gly	Phe	Leu	Ala
Lysine (c) (moles/6×10^3 g.)	—	—	—	1	1	1
Histidine (c)	—	—	—	2	2	2
Arginine (d)	—	1–2	1–2	1	1	1
Tyrosine (e)	2	2	2	2	1–2	1–2

1) Uncorrected with the flow by electro-osmosis.

(a) See Fig. 3; (b) See Fig. 2; (c) DNP-method (See Experimental part); (d) Spectrophotometry of the hydrolysate with Sakaguchi reaction (*15*); (e) Determined by direct spectrophotometry at 275 mμ.

* The isoelectric point was slightly higher than that of mammalian insulins (*1*).

and alanyl chains, were Sakaguchi reaction positive.

The isolation of glycyl and alanyl (or leucyl) chains in preparative scale, was achieved very efficiently by the fractional precipitation of the latter component with trichloroacetic acid, leaving the former component in the supernatant (*12*) (see, Fig. 3). The important properties of oxidized glycyl, alanyl and leucyl chains are summarized in Table I, together with those of oxidized beef insulin A and B.

DISCUSSION

Bonito insulin was found to be a mixture of two different components, which could be separated by elution chromatography on CM-cellulose with the use of concentration gradient of formic acid from $N/5$ up to N. According to Honma and Yamamoto,* the chromatographic system was also effective for the purification of other fish and mammalian insulins from the crude preparation.

Bonito insulin I was composed of glycyl and leucyl chains, while the other (II) had glycyl and alanyl chains. The both glycyl chains (having arginine) had an electromobility and a partition coefficient quite different from those of oxidized beef insulin A, while the oxidized leucyl and alanyl chains and beef insulin B were similar to each other in these respects. Some characteristic differences observed between the properties of insulins from bonito and from beef, such as the paper chromatographic R_f and the electromobility (*1*), may be mainly due to the structural difference in the former glycyl chain. In this connection, it should be mentioned that species differences among the chemical structure of mammalian insulin had been also observed only in this chain (*5*).

It will be of interest that bonito insulin II isolated from other protein, was fairly soluble in water at the isoelectric point, differing from that in crude preparation. As already mentioned, bonito insulin had been purified finally by the fractional precipitation from aqueous solution at the isoelectric point in a good yield (compare the ratio of insulin I and II in crude and in partially purified preparation, from the elution diagram shown in Fig. 1), but the further repeats of precipitation at the isoelectric point to obtain a crystalline preparation, had been accompanied with much loss of insulin-activity in the supernatant. The latter facts will be due to the fractional precipitation of insulin I.

The problem whether the two different insulins were derived from Langelhans islet of an individual bonito fish or from different fishes in the same species, will be very important. In the case of clupeines having alanine or proline as the N-terminal, the two different types, alanyl- and prolyl- clupeines were found to co-exsistent in sperm of a single fish (*Clupea pallasii*) (*16*). In the case of bonito fish, too, the authors** found that com-

* Personal communication.

** Unpublished

mercial insulin (partially purified one, see Scheme I) extracted from bonito fishes which had been captured at various part of the South-West Pacific Ocean, and at a different time (1955 58), possessed equally three different N-terminals, glycine, alanine and leucine at a same ratio of 0.9–1.0, 0.35–0.45, and 0.50–0.60 mole per 6×10^3 g., respectively.

The results would seem to suggest that the heterogeneity of bonito insulin seems to be a nature, characteristic and intrisic to the protein. However it could not be excluded form the consideration that Langelhans islet used in these studies, though freshly isolated from fish, had been kept in one per cent picric acid solution at 0 to 5° for some weeks before the extraction, and some limited proteolysis of an original insulin during the storage of gland, might result into the isolation of two different types of insulin. The isolation of insulin from fresh Langelhans islet of bonito fish, are now attempted.

There have been several attempts to isolate an fragment having insulin-activity from the proteolytic hydrolysate of beef insulin (*17*, *18*). In these respects, too, the study on the chemical structure of bonito insulin will be of interesting. As elucidated by the present authors, the structure of the both insulins from mammal and from fish seemed to be fairly different, so the identification of peptide fragments having common structure, might give some knowledge on the active center of the hormonal activity. The studies are now preceeding.

SUMMARY

1. Partially purified insulin from Langelhans islet of bonito, was separated on a column of CM-cellulose into two components having full insulin-activity; and that the one had one glycyl and one leucyl chains, while the other had one glycyl and one alanyl chains per 6×10^3 g. as the N-terminals, respectively.

2. It was described some evidences that the leucyl and alanyl chains present in bonito insulins I and II, corresponded to the phenylalanyl chain in mammalian ones, and some differences observed between the properties of both insulins from fish and from mammal seemed to be mainly due to the difference of the both glycyl chains.

The authors are greatly indebted to the Shimizu Seiyaku Co., Ltd., for the gifts of various kind of insulin. This study was in part supported by a grant-in-aid from the Ministry of Education.

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HEAT DECOMPOSITION PRODUCT OF FLAVIN ADENINE DINUCLEOTIDE IN AQUEOUS SOLUTION

III. CHEMICAL STRUCTURE OF "FOURTH FLAVIN COMPOUND"

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(Received for publication, January 27, 1960)

In the previous papers (*1*, *2*, *3*), it was reported that FAD is decomposed to FFC in aqueous solution by heating, and that this flavin compound, FFC, is converted to FMN by heating in acetic acid.

The physico-chemical properties of FFC were found to be quite similar with those of FMN.

From these results, it was considered that FFC is an intermediate product of hydrolysis from FAD to FMN and that the chemical structure of FFC is quite similar with that of FMN.

In the present paper, the chemical structure of FFC was studied and the results are reported in detail. The preliminary note had appeared (*4*).

MATERIALS

FFC—It was prepared and crystallized by the method described in the previous report (*2*).

FMN and *FR*—Chromatographically purified from commercial samples.

RESULTS AND DISCUSSIONS

Components of FFC—As reported previously (*1*), it was found that FFC converted to FMN by heating it in acetic acid.

In the present study, the hydrolysis of FFC by hydrochloric acid was examined.

It was found that FFC was converted quantitatively to FMN by heating it in *N* hydrochloric acid for 7 minutes in the same way as the measurement of $\Delta 7$ P. After the hydrolysis, the hydrolysate was chromatographed on filter paper and the absorption of ultraviolet light of 260 mμ was examined. The spot of adenosine or adenosine derivative was not found.

The phosphorus content of FFC was estimated by the method of Fiske-Subbarrow after the mineralization using sulfuric acid. The amount of

Abbreviations: FAD, flavin adenine dinucleotide; FFC, fourth flavin compound; FMN, flavin mononucleotide; FR, free riboflavin.

isoalloxazine nucleus in FFC was determined by the lumiflavin fluorescence method (*5*).

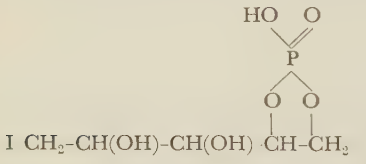
The result indicated that equimoles of isoalloxazine nucleus and phosphorus were contained in FFC. Considering the above-mentioned result and the previously reported result that the fluorescence intensity is not changed when FFC converted to FMN (*3*), it may be considered that FFC consists of equimoles of FR and phosphoric acid in a form which it can be easily changed to FMN.

Dissociation of Phosphoric Acid of FFC—It was reported in the previous paper (*3*) that the mobility of FMN in paper electrophoresis changed with increasing pH above 7.0, whereas FFC did not. This result indicates that FFC has not a second dissociation of phosphoric acid.

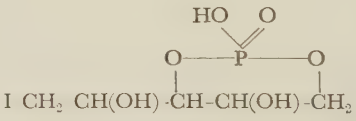
Considering the results mentioned before, the possible chemical structure for FFC will be as follows:

(I) riboflavin-4',5'-cyclic phosphate (*6*)
(II) riboflavin-3',5'-cyclic phosphate
(III) riboflavin-2',5'-cyclic phosphate or
(IV) diriboflavin pyrophosphate (*7*).

These compounds are composed of equimoles of riboflavin and phosphoric acid, and can produce FMN by hydrolysis. Compound (I) had been found by Forrest and Todd (*6*) in the solution of FAD treated with concentrated ammonia at room temperature.



(I)



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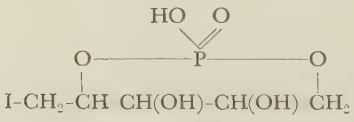

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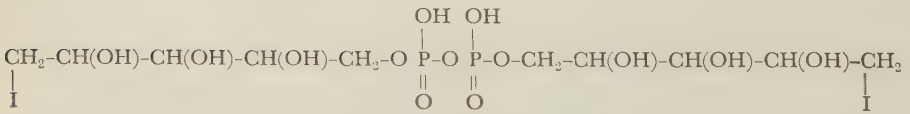

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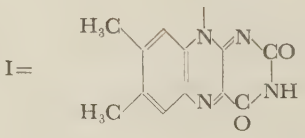


Periodate Oxidation of FFC—To identify the real structure of FFC with one of the above-mentioned supposed structures, periodate oxidation was practiced. The periodate consumption of flavin compound per mole of its isoalloxazine nucleus is theoretically expected as follows: one mole for (I) or (III), zero mole for (II), and two moles for (IV).

As the experiment, 1.0 ml. of 0.02 *M* $NaIO_4$ was mixed with 1.0 ml. of 0.005 *M* FFC solution (calculated on the basis of isoalloxazine nucleus), and placed in ice box. After the reaction, 0.2 ml. of diluted HCl and 1 ml. of KI were added and iodine liberated was titrated by 0.02 *M* sodium thiosulfate (*8*, *9*).

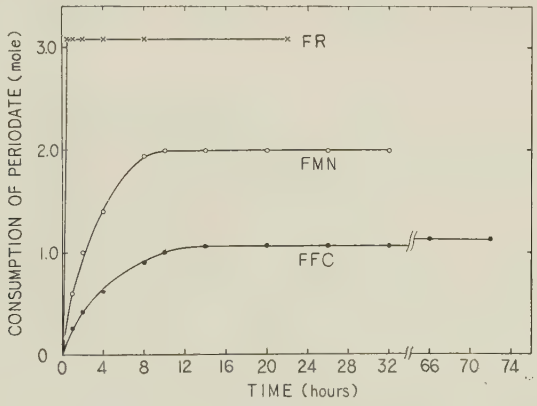


Fig. 1. Consumption of periodate by flavins.
Mole of periodate consumed per mole of flavin calculated on the basis of isoalloxazine nucleus after the indicated period of reaction was shown.

As shown in Fig. 1, it took about 72 hours for complete oxidation of FFC by periodate. The consumption of periodate by one mole of FFC was 1.13 moles.

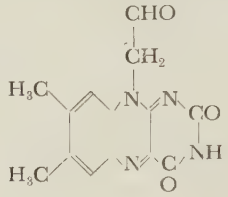
As the controls of experimental condition, FR and FMN were also studied. In the case of FR, 1 mole of FR consumed 3.08 moles of periodate after 30 minutes, no more periodate consumption was observed even after 22 hours (the theoretical value is 3.0 moles). In the case of FMN, 10 hours were necessary for its complete oxidation by periodate, and the consumption of periodate was 1.99 moles (the theoretical value is 2.0 moles). Even 30 hours later, further consumption of periodate was not observed.

From these results, the possible structure of FFC could be restricted to (I) or (III), one mole of which consumes one mole of periodate.

To identify the structure of FFC with (I) or (III), the oxidized product was further examined.

Periodate Oxidation Product of FFC and Other Flavins—The oxidized products were examined by paper chromatography using 5 per cent disodium hydrogen phosphate as the mobile phase.

When irradiated by ultra-violet ray of 365 mμ, in all cases, *i.e.*, FFC, FMN or FR, yellow fluorescent spot appeared at R_f 0.13. This result indicates that the same oxidized product having isoalloxazine nucleus might be produced in the above-mentioned three cases. Theoretically, FR gives 6,7-dimethyl-isoalloxazine-9-acetaldehyde (V) as the oxidized product by periodate.



(V)

Wickström (*10*) had described the theoretical occurrence of this compound from FR by periodate oxidation, but he did not isolate this compound.

To identify the oxidized product of FFC with that of FR, the isolation and identification of the oxidized product was further examined.

Isolation of Oxidized Product of FFC—For the separation of this oxidized product, 0.05 mmoles of FFC were dissolved in 3 ml. of distilled water and mixed with 2 ml. of 0.07 m*M* of periodate, and stored in ice box for 60 hours. The crystals of plate form were obtained as shown in Fig. 2. The formed crystals were filtered, and washed with cold water.

Fig. 2. Periodate oxidation product of FFC, magnified 400 times.

About 14 mg. of orange yellow crystals was obtained. This crystal was insoluble in ether, hardly soluble in chloroform, slightly soluble in water and alcohol, and freely soluble in phenol. This sample reduces the Tollens' reagent at room temperature, and the Fehling's solution on a boiling water

bath. In the dilute aqueous solution, this compound is unstable, especially for light irradiation, namely, it is photodecomposed to lumiflavin in alkaline medium, and to lumichrome in acidic medium. The absorption spectrum of this compound in water has the peaks at 266, 372 and 445 mμ, as shown in Fig. 3.

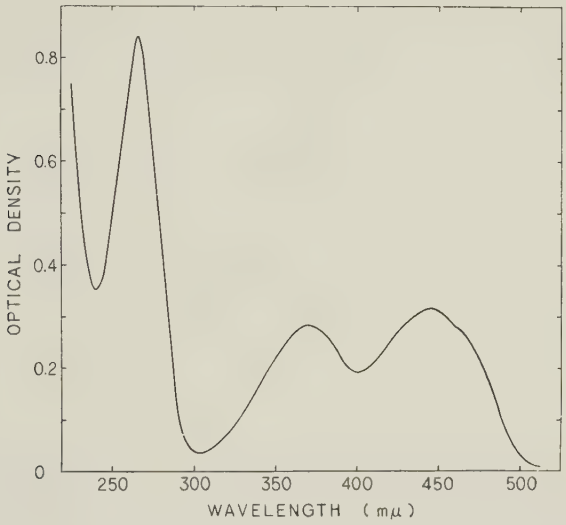


Fig. 3. Absorption spectrum of periodate oxidation product (6,7-dimethyl-isoalloxazine-9-acetaldehyde) of FFC, aqueous solution.

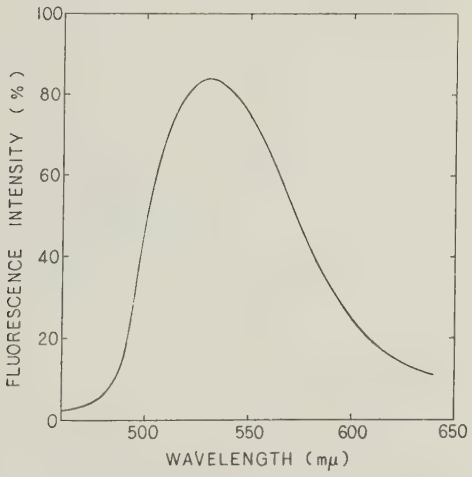


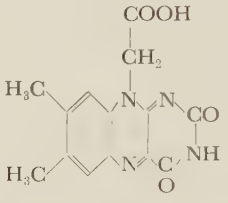
Fig. 4. Fluorescence spectrum of periodate oxidation product (6,7-dimethyl-isoalloxazine-9-acetaldehyde) of FFC, aqueous solution.

The peak of fluorescencence spectrum is recorded at 530 mμ as same as the other flavin compounds, as shown in Fig. 4.

Considering these properties, it seems that this compound contains isoalloxazine ring and aldehyde group which has reductability. These results show that this compound may possibly be considered to be 6,7-dimethyl-isoalloxazine-9-acetaldehyde.

Chemical Structure of Periodate Oxidation Product of FFC—As the compound is unstable, as mentioned before, the further identification was practiced by using its derivative.

A small amount of bromine water was added to aqueous solution of this compound, then stood for 12 hours in dark room. The yellow substances were collected by phenol extraction method and identified with 6,7-dimethyl-isoalloxazine-9-acetic acid (VI) by paper chromatography.



(VI)

Fig. 5. Crystals of 6,7-dimethyl-isoalloxazine-9-acetic acid methyl ester, magnified 500 times.

The R_f values are 0.45 (mobile phase: 5 per cent Na_2HPO_4) and 0.43 (mobile phase: the upper layer of *n*-butanol, acetic acid, water mixture, 4 : 1 : 5).

Then, this powder was dissolved in dried methanol and saturated with dried hydrochloric acid gas, stored at room temperature for 12 hours, and added to 5 times its volume of water, and extracted with chloroform. The chloroform layer was washed several times with water, then the chloroform

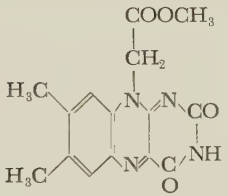
layer was evaporated and the residue was recrystallized from ethyl alcohol aqueous solution. The yellow crystals of needle form were obtained as shown in Fig. 5.

The R_f values of this derivative were 0.63 (mobile phase: the upper layer of *n*-butanol, acetic acid, water mixture, 4 : 1 : 5), and 0.4 (mobile phase: 5 per cent Na_2HPO_4). It melts at the range of 288-293° with decomposition. These properties are identical with those of 6,7-dimethyl-isoalloxazine-9-acetic acid methyl ester, which had been reported by Fukamachi *et al.* (*11*, *12*).

The result of elemental analysis of the crystals of this methyl ester was as follows:

	C%	H%	N%
calculated for $C_{15}H_{14}N_4O_4$	57.31	4.49	17.83
found	57.23	4.66	17.93

So, the methyl ester derived from the periodate oxidation product of FFC was identified with 6,7-dimethyl-isoalloxazine-9-acetic acid methyl ester (VII).



(VII)

Periodate oxidation products of FR and FMN were also obtained in the same way as described above. And the same results as FFC were obtained.

Consequently, the periodate oxidation product of FFC is determined to be 6,7-dimethyl-isoalloxazine-9-acetaldehyde (V).

These results indicate that the cleavage of ribitol chain of FFC takes place between C_2' and C_3' by periodate oxidation. Thus, the structure of FFC was identified with (I), riboflavin-4′,5′-cyclic phosphoric acid (*6*).

Finally, the elemental analysis was practiced for FFC, the result being summarized as follows:

	C%	H%	N%	O%	P%
calculated for $C_{17}H_{19}N_4O_8P$	46.58	4.37	12.78	29.20	7.07
found	46.71	4.48	12.70	29.06	7.05

This result confirmed the above-mentioned chemical structure of FFC. The result reported by Forrest and Todd (*6*) that riboflavin-4′,5′-cyclic phosphoric acid is produced from FAD by ammonia treatment might be analogical as alkaline hydrolysis of nucleic acid. However, present result that riboflavin-4′,5′-cyclic phosphoric acid can also be produced from FAD by simple heating of its neutral aqueous solution (*2*), should be noted concerning the chemical properties of FAD.

SUMMARY

Chemical structure of FFC was studied. The components of FFC were determined to be equimoles of riboflavin and phosphoric acid. Considering the previous result that FFC has not a second dissociation, of phosphate group, the chemical structure was supposed to be riboflavin cyclic phosphate. Then, periodate consumption was estimated, and it was found that one mole of periodate was consumed per one mole of isoalloxazine nucleus of FFC. The periodate oxidation product of FFC was analyzed. When this product was oxidized by bromine water, it gave 6,7-dimethyl-isoalloxazine-9-actic acid, methyl ester of which was further identified with 6,7-dimethyl-isoalloxazine-9-acetic acid methyl ester. Thus, the periodate oxidation product was found to be 6,7-dimethyl-isoalloxazine-9-acetaldehyde. From these results, the cleavage of ribitol chain of FFC by periodate oxidation was found to take place between C_2' and C_3'. Consequently, the structure of FFC was identified with riboflavin-4′,5′-cyclic phosphoric acid which had been found by Forrest and Todd in the solution of FAD treated with concentrated ammonia.

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TREHALASE OF THE SILKWORM, BOMBYX MORI

PURIFICATION AND PROPERTIES OF THE ENZYME

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Recently it has been shown by several workers (*1*, *2*) that the non-reducing disaccharide trehalose is a major blood sugar in a number of insect species. Their findings have induced much interest on the metabolism of this sugar. About two decades ago, Frèrejacque (*3*) found that enzyme preparations from several insects can split trehalose much more rapidly than sucrose or maltose. More recently, Howden and Kilby (*2*) have reported that the blood plasma and tissues of the locust, *Shistocerca gregaria* contain an enzyme system capable of hydrolyzing trehalose in addition to maltose, sucrose *etc.*

None of the above workers, however, has purified the enzyme. Since it is more desirable to investigate the properties of the enzyme with more purified preparation, the present work has been undertaken employing an ion-exchange chromatography for enzyme purification. During the course of this work, Kalf and Rieder (*4*) have reported the purification and some properties of a specific trehalase from the larvae of the wax moth, *Galleria mellonella*.

The present paper deals with the purification and characterization of the enzyme trehalase from the silkworm, *Bombyx mori*.

EXPERIMENTALS

Silkworm pupae were used as materials for the purification of the enzyme. To investigate the distribution of the enzyme, fully grown larvae of the 5th instar were employed. Trehalose and other sugars were purchased from Wako Pure Chemical Industries, Tokyo. Methyl α-D-glucoside (*5*) and phenyl α-D-glucoside (*6*) were synthesized. Diethylaminoethyl (DEAE)-cellulose used for chromatography was kindly supplied by Mr. M. Yamamoto of this university.

Trehalase activity was assayed by determining the rate of enzymic release of glucose from trehalose. Unless otherwise stated, the reaction mixture contained 0.5 ml. of 0.02 *M* trehalose, 0.5 ml. of McIlvaine's citrate-phosphate buffer, pH 5.2, and 0.5 ml. of the enzyme solution, in a final volume of 2 ml. Reaction was initiated by adding either the enzyme or the substrate. After incubation for 15 minutes at 30°, the reaction mixture was immersed in a boiling water bath for 3 minutes and immediately cooled with running tap water. The coagulated protein was centrifuged off and an aliquot of the super-

natant was analyzed for glucose by arsenomolybdate meteod (*7*). Under the above conditions the rate of glucose release was proportional to the enzyme concentration and it was held constant for more than one hour (Fig. 1).

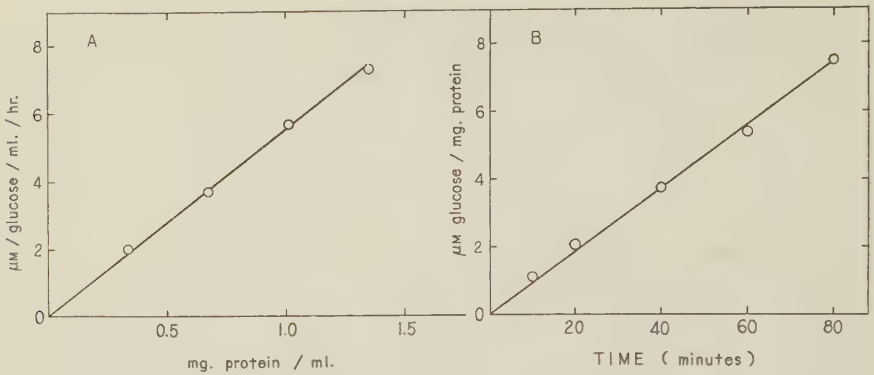

Fig. 1. Proportionality of glucose release to enzyme concentration (A) and linearity of glucose release with incubation time (B).

In both A and B, crude extract was used as enzyme solution. Substrate concentration 0.005 *M*; pH 5.2; 30°.

A unit of enzyme was defined as the amount of the enzyme which would liberate one μmole of glucose from trehalose in one hour under the standard conditions. The specific activity of the enzyme was expressed in terms of the enzyme units per mg. of protein which was estimated with Folin-Ciocalteu reagent as described by Lowry *et al.* (*8*) using egg albumin as the standard. As the measure of protein concentration of chromatographic effluent, the optical density at 280 mμ was determined with Hitachi spectrophotometer Model EPU-2A.

RESULTS

1. Purification of the Enzyme

The procedure for the purification of trehalase consisted essentially of four operations: preparation of acetone powder from silkworm pupae; extraction of the enzyme from the acetone powder with dilute acetic acid; fractionation with ammonium sulfate, and resolution of the proteins by DEAE-cellulose column chromatography. Unless otherwise stated, all operations were carried out in a cold room (approximately 2°).

Purification protocols are shown in Table I.

Preparation of Acetone Powder—Silkworm pupae were homogenized in a Waring Blendor for 3 minutes and the homogenate was left for 30 minutes with occasional shaking. After centrifugation for 5 minutes at 4,000 r.p.m., middle layer was put into 15 volumes of acetone cooled to −15°. The mixture was filtered on Büchner funnel and the residue was dried *in vacuo* over $CaCl_2$. Acetone powder thus prepared was very stable and could be stored at least for two years at 0–2° without appreciable loss of the enzymic activity.

Extraction from Acetone Powder—Ten g. of acetone powder were homogenized with 160 ml. of cold 0.2 *M* acetic acid containing thiourea (0.002 *M*) in Potter-Elvehjem homogenizer. The homogenate was allowed to stand

TABLE I

Purification of Trehalase from Silkworm Pupae

Purification step	Total volume	Total protein	Total activity	Specific activity	Yield
	ml.	mg.	unit	unit/mg. protein	%
Crude extract	152	4,099.4	17,863	4.3	(100)
Dialyzed fraction	13.3	425.3	9,185	21.6	51.5
Chromatographic effluent					
fraction 38-56	190	16.96	4,938	292	27.7
fraction 42	10	1.48	548	368	—
fraction 50	10	0.40	404	1,010	—

for 60 minutes with continuous stirring and then centrifuged in a Kubota refrigerated centrifuge for 15 minutes at 8,000×*g*. The resulting supernatant (crude extract in Table I) was used for further purification.

Ammonium Sulfate Fractionation and Dialysis—One hundred and seventyfive ml. of ammonium sulfate solution saturated at 0° were added to 152 ml. of crude extract, and pH of the mixture was adjusted to 7.7 by adding 4 *N* NH_4OH. After standing for 60 minutes the precipitate was removed by centrifuging for 15 minutes at 15,000×*g*. The pH of supernatant was then adjusted to 4.5 by adding 5 *N* H_2SO_4. The precipitate which formed on standing at pH 4.5 for 120 minutes was collected by centrifugation at 20,000×*g* for 20 minutes and dissolved in 8 ml. of cold water. The solution was dialyzed overnight against tap water (2-4°) and the precipitate which appeared on dialysis was removed by centrifuging at 15,000×*g* for 15 minutes. To 11.3 ml. of the supernatant, 2 ml. of 0.067 *M* phosphate buffer (pH 6.0) were added to give a phosphate concentration of 0.01 *M* (dialyzed fraction).

Chromatography on DEAE-cellulose Column—DEAE-cellulose column (2.2×9 cm.) was prepared as described by Sober *et al.* (*9*) and equilibrated wtih 0.01 *M* phosphate buffer (pH 6.0). Eleven ml. of dialyzed fraction containing 352 mg. of protein were run onto the column. One hundred and sixty ml. of 0.05 *M* KH_2PO_4 was passed and then the gradient elution was started by introducing 0.05 *M* KH_2PO_4-0.1 *M* NaCl from upper reservoir into the mixing chamber which initially contained 150 ml. of 0.05 *M* KH_2PO_4. Fractions of 10 ml. volume were collected at the rate of about 18 ml. per hour. A typical chromatogram was presented in Fig. 2. It can be seen from the figure that the enzymic activity showed two peaks. Another chromatogram, which was

obtained by a somewhat different procedure*, similarly showed two peaks. These results suggest the presence of the two forms of trehalase, which may be tentatively designated as component A and B, the former being the one eluted earlier. In Fig. 2, the peaks of optical density at 280 mμ corresponding to trehalase activity were not seen, owing to the extremely low concentration of the proteins in the effluents. The specific activities of the fraction 42 and 50 were very high, indicating that in these fraction the enzyme had been purified 85 and 230 fold, respectively.

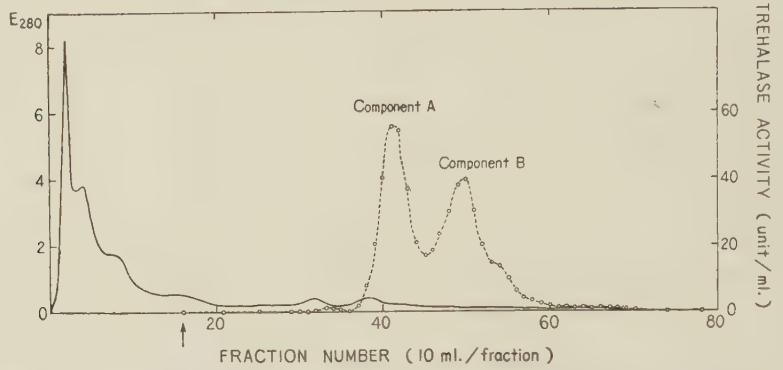

FIG. 2. DEAE-cellulose chromatography of dialyzed fraction.
The experimental condition is given in the text. The gradient elution was started at the fraction indicated by the arrow.
--○--: trehalase activity under standard condition.
——: optical density at 280 mμ.

2. *Properties of the Purified Enzyme*

In the following experiments, component A(fraction 42) and component B(fraction 50) with appropriate dilution were used throughout.

Optimum pH—The enzymic hydrolysis of trehalose was studied as a function of pH with McIlvaine's citrate-phosphate buffer. As shown in Fig. 3, an optimum pH of 5.2 was found for both components.

Michaelis Constant—The effect of substrate concentration on the catalytic activity of the two fractions was determined at pH 5.2. A typical Michaelis-Menten relationship was obtained. From double reciprocal plots (Fig. 4), the values of K_m were calculated to be 4.4×10^{-4} *M* for component A and 4.7×10^{-4} *M* for component B.

Effect of Temperature on the Reaction Rate—The effect of temperature on the rate of enzymic hydrolysis of trehalose was examined over the range 14–37°. The results were plotted in the conventional Arrhenius manner

* In this case, the protein fraction precipitating between 0.37–0.55 ammonium sulfate saturation at pH 4.4 was used. After being treated as described above, the protein was adsorbed on the DEAE-cellulose column and eluted by passing 0.07 *M* KH_2PO_4-0.07 *M* NaCl.

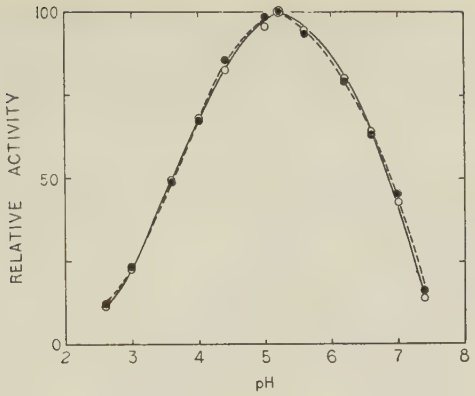

Fig. 3. The effect of pH on the trehalase activity. Entire range of pH was covered with McIlvaine's citrate-phosphate buffer. Each tube contained 10 μmoles of trehalose, 0.5 ml. of the buffer, and 18.5 μg. enzyme protein (component A) or 5 μg. enzyme protein (component B), in a final volume of 2.0 ml.

—○—: component A --●--: component B

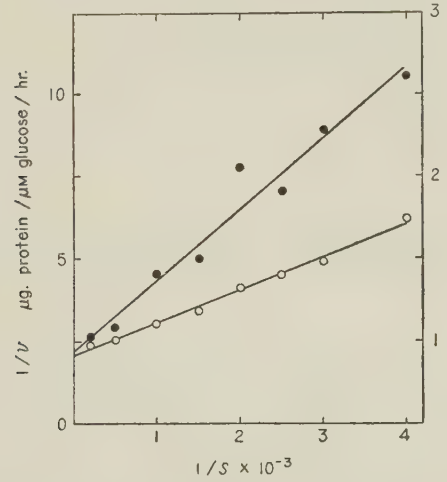

Fig. 4. The effect of substrate concentration on the rate of its enzymic hydrolysis.

Each tube contained 0.5 ml. of the buffer (pH 5.2), 6.75 μg. enzyme protein (component A) or 1.83 μg. enzyme protein (component B), and indicated concentration of trehalose, in a final volume of 2.0 ml.

S: molar concentration of trehalose in the reaction mixture.

v: rate of glucose release in μmoles per μg. protein per hour.

The right ordinate for component B (—●—), the left ordinate for component A (—○—).

as shown in Fig. 5. The average activation energies for component A and B were 9.4×10^3 cal. per mole and 9.7×10^3 cal. per mole, respectively.

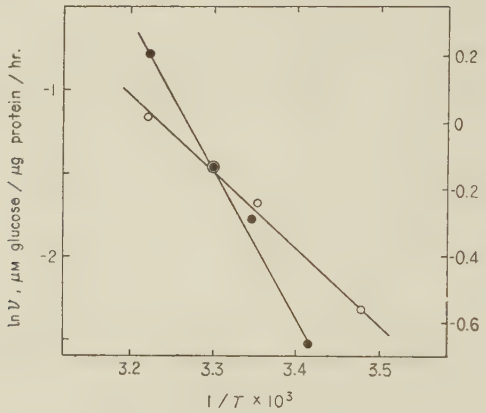

Fig. 5. An Arrhenius plot for the enzymic hydrolysis of trehalose.

The composition of reaction mixture was the same as for Fig. 3.

T: absolute temperature.

v: rate of glucose release in μmoles per μg. enzyme protein per hour.

The right ordinate for component B (—●—), the left ordinate for component A (—○—).

Substrate Specificity—Under the condition of the standard assay method, no hydrolysis of the following glycosides could be demonstrated: sucrose, maltose, salicin, lactose, cellobiose, methyl α-D-glucoside, phenyl α-D-glucoside and starch.

Stoichiometry—As illustrated in Table II, each μmole of trehalose yields two μmoles of glucose. When phenylhydrazine and sodium acetate were added to the reaction mixture after the completion of hydrolysis, typical crystals of phenylglucosazone were obtained.

Survery of Activator and Inhibitor—So far as examined, no activator or inhibitor could be found. Following compounds at the indicated final concentrations did neither accelerate nor inhibit enzymic hydrolysis of trehalose: monoiodoacetic acid (10^{-3} *M*), ethylenediaminetetraacetic acid (2×10^{-3} *M*), aniline (10^{-3} *M*), $MgCl_2$ (5.1×10^{-3} *M*), $CaCl_2$ (5.1×10^{-3} *M*), glucose (4×10^{-3} *M*), methyl α-D-glucoside (1.3×10^{-2} *M*), cysteine (7.3×10^{-3} *M*), dopa-melanin (12.5 μg./ml.)*, and protamine sulfate (125 μg./ml.)*.

* These compounds were preincubated with the enzyme for 10 minutes at 30° before addition of the substrate.

3. The Distribution of Trehalase in the Silkworm Larval Tissues

In order to examine the distribution of trehalase in the body, several tissues listed in Table III were dissected out from 5th instar larvae and

TABLE II

Stoichiometry of Trehalase Reaction

Incubation time	Trehalose added	Glucose found	
		component A	component B
min.	μmole	μmole	μmole
190	0.104	0.213	0.210
190	0.208	0.426	0.433

The indicated amounts of trehalose were incubated in the reaction mixture containing 0.5 ml. of McIlvaine's buffer (pH 5.2), 29.6 μg. of enzyme protein (component A) or 8 μg. of enzyme protein (component B), in a final volume of 2.0 ml.

washed in Ringer solution. The tissues were homogenized in Potter-Elvehjem homogenizer with appropriate volume of dilute McIlvaine's buffer (pH 5.2) and the homogenate was allowed to stand for 30 minutes at 0°. After centrifugation at 3,500 r.p.m. for 5 minutes clear supernatant was used as enzyme solution. Body fluid was collected as described by Kuwana (*10*), and thiourea at final concentration of $2\times10^{-3}\,M$ was added to prevent melanization.

The results were shown in Table III. Intestine showed extremely high enzymic activity. No activity could be found in body fluid and silk gland.

TABLE III

The Distribution of Trehalase in the Tissues of 5th Instar Silkworm Larvae

Tissue	Trehalase activity
	units/mg. protein
Body fluid	0.0
Silk gland	0.0
Muscle from body wall	0.28
Fat body	0.58
Intestine	11.84

Trehalase activity of supernatant from tissue homogenates was measured under the standard condition. The activity was expressed in terms of units per mg. protein of tissue extract or body fluid.

DISCUSSION

The enzyme trehalase is widely distributed among microorganisms, fungi, and animals (*11*, *12*). In 1941, Frèrejacque (*3*) reported that preparations from *Gryllotalpa gryllotalpa* and *Doryphora sp.* hydrolyze trehalose, sucrose, and maltose. Similar results were obtained by Howden and Kilby (*2*) in the locust, *Schistocerca gregaria*, and Zebe and McShan (*13*) in the woodroach, *Leucophaea maderae*. It is apparent from the present work together with that of Kalf and Rieder (*4*) that the enzyme is not a α-glucosidase in a narrow meaning, but a specific one for trehalose.

By employing DEAE-cellulose chromatography in the purification, the specific activity of the resulting preparation was elevated 25 times as that obtained by Kalf and Rieder. The effluents of chromatography had two peaks of trehalase activity, suggesting the presence of two forms of trehalase. However, no difference was observed in their properties so far as trehalose hydrolysis is concerned. Probably they may differ in minor structural details, such as the number of carboxyl groups as described on ribonuclease A and B by Tanford and Hauenstein (*14*).

Little is known about the physiological role of trehalase. As seen in Table III, the trehalase activity of fat body is relatively low. Extremely high activity was found in the intestine. These results were essentially similar to that of Zebe and McShan (*13*) in woodroach. Trehalose synthesis by the reverse action of trehalase was observed under the presence of 20 per cent glucose (*13*). Such a condition, however, is not a physiological one, and the major role of the enzyme would be the hydrolysis.

Trehalose of silkworm body fluid rapidly disappeared on starvation (*15*), suggesting that it might be used as an energy source. Wyatt and Kalf (*1*) suggested the participation of a phosphorylase in the metabolism of trehalose. But in author's preliminary experiments with extracts from silkworm intestine and fat body, the presence of such a phosphorylase was excluded and it was concluded that glucose released from trehalose by the action of trehalase enters into the glycolytic pathway followed by phosphorylation with adenosinetriphosphate.

The detailed studies on the role of trehalose and trehalase will be reported elsewhere.

SUMMARY

1. From the pupae of the silkworm, *Bombyx mori*, an enzyme which specifically hydrolyzes trehalose, has been purified.

2. Chromatographic procedure has revealed the existence of two forms of trehalase, but their properties did not differ as fas as trehalose hydrolysis is concerned.

3. In a larval stage, trehalase activity is found in intestine, body-wall muscle, and fat body, but not in body fluid and silk gland.

The author wishes to express his gratitude to Prof. Y. Akita and Assist. Prof. M. Akino for their guidance and criticism throughout this work. Thanks are also due to Prof. K. Dan for reading the manuscript.

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GLUTAMIC ACID FORMATION FROM GLUCOSE BY BACTERIA

IV. CARBON DIOXIDE FIXATION AND GLUTAMATE FORMATION IN BREVIBACTERIUM FLAVUM NO. 2247

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In the preceding papers of this series (*1–3*). in which glutamic and α-ketoglutaric acid formation in resting cells and cell-free extracts of *Brevibacterium flavum* were dealt with, several remarkable features of this bacterium were described. First, enzymes of glycolysis, Krebs cycle, and "glyoxylate bypass" (*4*) were found to be present in the cell-free extract. Secondly, pathway of pyruvic acid formation from glucose exists in this bacterium. The results thus far obtained showed that pyruvic acid was formed from glucose primarily through the Embden-Meyerhof pathway.

Since the work on heterotrophic carbon dioxide fixation by Wood and Werkman (*5*) had been published, the formation of OAA* from pyruvic acid and CO_2 was considered as a key reaction in the tricarboxylic acid cycle and hence for the formation of many amino acids including glutamic acid. Later, however, there have been described five enzymes or enzyme systems that form dicarboxylic acid of the cycle from three carbon compound and CO_2. These are; (a) OAA carboxylase found in pigeon liver by Utter and Kurahashi (*6–8*), which catalyzes the reversible carboxylation of PEP to form OAA in the presence of IDP. (b) PEP carboxylase discovered by Bandurski in spinach leaves (*9*, *10*), which synthesizes OAA from PEP and CO_2 without requiring any nucleoside polyphosphate as a phosphate acceptor. (This reaction is not reversible.) (c) A reversible type of OAA decarboxylase found first in *Micrococcus lysodeikticus* (*11*, *12*), which can catalyze an incorporation of CO_2 in OAA, and does not require ATP or ITP (*13*), but requires orthophosphate (*14*). (d) "Malic" enzyme of Ochoa (*15*, *16*) catalyzing the reversible oxidation of malate to pyruvate and CO_2 in the presence of TPN. (e) An enzyme system catalyzing the decarboxylation of succinate, which can produce succinate from propionate and CO_2 in the presence of ATP and CoA (*17*, *18*).

* The abbreviations used through this paper include OAA, oxaloacetic acid; PEP, phosphoenolpyruvic acid; TPN, triphosphopyridine nucleotide; IDP, inosine diphosphate; ATP, adenosine triphosphate; ITP, inosine triphosphate; CoA, coenzyme A; GSH, reduced glutathione; Tris, tris (hydroxymethyl) aminomethane; cpm., counts per minute.

A preliminary experiment with the whole cells of *Brevibacterium flavum* revealed a considerable fixation of $C^{14}O_2$ in the α-carboxyl carbon of glutamic acid formed during the oxidation of glucose or some other substrates. This led us to investigate, using cell-free extracts, the enzymic systems responsible for the CO_2 fixation.

METHODS

Chemicals—Oxaloacetic acid, pyruvic acid, L-malic acid, TPN, GSH, ATP, and CoA were purchased from California Corporation for Biochemical Research. ITP and $BaC^{14}O_3$ were obtained from Nutritional Biochemicals Corporation and the Radiochemical Centre, Amersham, respectively.

Washed Cell Suspension and Cell-free Extract—After growing the cells in the medium described previously (*1*) for 24 hours, they were harvested by centrifugation, washed with 0.2 per cent KCl, suspended in 0.05 *M* phosphate buffer, pH 7.5, aerated at 30° for one to two hours, and resuspended in 0.01 *M* phosphate buffer, pH 7.5. Cell-free ectract was prepared from dried cells. The washed cells were dried over P_2O_5 *in vacuo*. The dried cells were ground in a mortar, suspended in 0.01 *M* Tris buffer, and allowed to stand for two hours. The suspension was finally centrifuged at $18{,}000 \times g$ for 30 minutes.

Incorporation of C^{14} from $C^{14}O_2$ into Glutamate and α-Ketoglutarate Formed by Washed Cells—The Methods employed for the reaction and determination of specific activity and distribution of the labelling of glutamate formed were essentially the same as described previously (*19*), and the method for determination of specific activity of α-ketoglutarate and of radioactivity of CO_2 were also essentially the same as described in another previous paper (*3*). Carbon dioxide was measured manometrically.

C^{14}-Incorporation from CO_2 into Four-Carbon Dicarboxylic Acid by Cell-free Extract—Unless otherwise noted, the experiments were run in Warburg vessels with three side arms. 0.5 ml. of cell-free enzyme extract, 0.4 ml. of 0.025 *M* $NaHC^{14}O_3$, and 0.4 ml. of 0.5 *N* HCl were introduced in to each side arm. The main chamber contained all the other components. After temperature equilibration, enzyme preparation and $NaHC^{14}O_3$ in side arms were tipped in, the final pH becoming 7.5. At the end of the incubation period, the reaction was stopped by tipping in HCl. After CO_2 was removed by aeration, the reaction mixture was centrifuged and analysed as described in the previous papers (*3*, *19*). OAA was isolated after conversion to its 2,4-dinitrophenylhydrazone on a paperchromatogram, eluted with water, and dried *in vacuo*. The radioactivity of the sample was measured with G-M tube, and the amount of the hydrazone was determined spectrophotometrically in 1 *N* NaOH at 440 mμ. Other organic acids were isolated in free acid forms by paperchromatography (solvent: phenol-formic acid). Malic acid was assayed by the colorimetric method of Goodban and Stark (*20*).

RESULTS

Incorporation of C^{14} from $C^{14}O_2$ into Glutamate and α-Ketoglutarate Formed by Washed Cells—When *Brevibacterium flavum* is grown with glucose as the sole carbon source, a considerable amount of α-ketoglutarate, or L-glutamate depending upon the presence of NH_4^+ ions, is aerobically formed from glucose as well as from acetate, pyruvate, oxaloacetate, and succinate by the washed cells. The origin of glutamate and α-ketoglutarate may be ascribed to the reactions shown in Fig. 1.

According to Fig. 1, carbon dioxide is fixed in the β-carboxyl group of oxaloacetate, which subsequently enters the TCA cycle. If citrate is metabolized asymmetrically in the manner characteristic of aminal tissues (*21*),

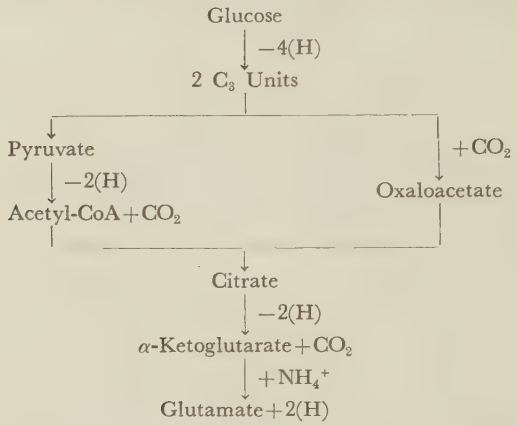

FIG. 1. Glutamate formation from glucose through the carboxylation of three carbon unit.

the isotope will appear predominantly in the α-carboxyl group of glutamate. Table I shows that the addition of C^{14}-carbonate to the reaction mixture leads to the formation of glutamate, in which the carbon atom of the α-carboxyl group has a specific activity about 100 times as great as the average

TABLE I

Incorporation of C^{14} from $C^{14}O_2$ into Glutamate Formed by Resting Cells of Brevibacterium flavum No. 2247

Substrate	Glutamate formed	Specific radioactivity		Percentage of counts in α-carboxyl-C
		Glutamate	CO_2 after reaction	
	μM	$\times 10^4$ cpm./μM		%
Glucose, 10	7.4	2.01	1.48	98.3
Acetate, 30	6.2	0.351	1.16	94.9
Pyruvate, 20	1.6	0.308	1.18	96.3
Glucose 10 + succinate, 10	9.5	1.84	1.05	98.5
Acetate 30 + succinate, 10	8.1	0.324	1.24	94.1
Pyruvate 20 + succinate, 10	5.2	0.336	1.17	97.3
Succinate, 20	0.7	—	—	—

Reaction mixtures contained 50 μM of phosphate buffer, pH 7.5, 10 μM of ammonium carbonate, 10^6 cpm. of C^{14}-sodium carbonate, substrate as indicated, washed cell suspension containing 0.65 mg. N., and water in a total volume of 1.6 ml. Shaken in Warburg vessels at 37° for 5 hours.

of that found in the other caron atoms. In this respect the result is consistent with the hypothesis. Moreover, the fact that the specific activity of glutamate derived from glucose is greater than that of carbon dioxide isolated after the reaction suggests the occurrence of net fixation of carbon dioxide, besides exchange reaction with some intermediates. On the other hand, low specific activity of glutamate derived from acetate and pyruvate can be explained in terms of the exchange reactions occurring in some intermediary steps. Similar results were obtained with α-ketoglutarate formations as shown in Table II.

TABLE II

Incorporation of C^{14} from $C^{14}O_2$ into α-Ketoglutarate Formed by Resting Cells of Brevibacterium flavum No. 2247

Substrate		α-Ketoglutarate formed	Specific radioactivity	
			α-Ketoglutarate	CO_2 after reaction
	μM	μM	$\times 10^4$ cpm./μM	
Glucose,	10	4.42	1.88	1.02
Acetate,	30	1.69	0.244	0.932
Oxaloacetate,	20	3.07	0.310	1.17
Pyruvate,	20	2.12	0.468	1.33
Succinate,	20	0.02	0.262	2.07

Reaction mixtures contained 50 μM of phosphate buffer, pH 7.5, 10 μM of sodium bicarbonate, 10^6 cpm. of C^{14}-sodium carbonate, substrate as indicated, washed cell suspension containing 0.40 mg. N., and water in a total volume of 1.6 ml. Shaken in Warburg vessels at 37° for 2.5 hours.

Incorporation of C^{14} from $C^{14}O_2$ into Four Carbon Dicarboxylic Acids by Cell-free Extracts—Since the results of the preceding section suggests the occurrence of enzymes catalyzing the carboxylation reaction to form four carbon dicarboxylic acid of the Krebs cycle, radioactive carbon dioxide was incubated with OAA, malate, and succinate, respectively, in the presence of cell-free extract of *B. flavum*. The radioactivity incorporated in these acids was determined as described previously. As shown in Table III, considerable radioactivities were fixed in OAA and malate isolated by paperchromatography, but not in succinate. Fumarate formed from malate by the action of fumarase in the cell-free extract (*1*) was also radioactive.

Because of the rapid decomposition of OAA by the cell-free extract, total radioactivity of residual OAA decreases after the maximum value is attained, while its specific activity increases continuously with time (Fig. 2).

Table IV shows clearly the dependence of the CO_2-OAA exchange reaction on the presence of ITP and manganous ions. These results can be taken as an evidence for the presence of reversible oxaloacetic carboxylase in the cell-free extract of *B. flavum*.

"Malic" enzyme was demonstrated in the cell-free extract by the reduc-

TABLE III

Incorporation of C^{14} from $C^{14}O_2$ into Four Carbon Dicarboxylic Acids by Cell-free Extract of Brevibacterium flavum No. 2247

Substrate	Incorporation into substrate (total count)	
Oxaloacetate[1]	11.4×10^2 cpm.	
,,	0.0 ,,	(into pyruvate[5])
Malate[2]	6.8 ,,	
,,	2.6 ,,	(into fumarate[5])
Succinate[3]	0.0 ,,	
Oxaloacetate[4]	24.2 ,,	

1) Reaction mixture contained 40 μM of OAA (K-salt), 5 μM of GSH, 3 μM of ITP, 2 μM of $MnSO_4$, 10 μM of $NaHCO_3$, 10^6 cpm. of C^{14}-sodium carbonate, 50 μM of phosphate buffer, pH 7.5, cell-free extract containing 0.38 mg. N., and water in a total volume of 1.6 ml. Incubated at 37° for 20 minutes.

2) Reaction mixture contained 20 μM of Potassium malate, 1 μM of TPN, 2 μM of $MnSO_4$, 10 μM of $NaHCO_3$, 10^6 cpm. of C^{14}-sodium carbonate, 50 μM of phosphate buffer, pH 7.5, cell-free extract containing 0.38 mg. N., and water in a total volume of 1.6 ml. Incubated at 37° for 20 minutes.

3) Reaction mixture contained 20 μM of potassium succinate, 0.08 μM of CoA, 5 μM of ATP, 10 μM of $MgCl_2$, 10 μM of $NaHCO_3$, 10^6 cpm. of C^{14}-sodium carbonate, 50 μM of phosphate buffer, pH 7.5, cell-free extract containing 0.38 mg. N., and water in a total volume of 1.6 ml. Incubated at 37° for 20 minutes.

4) Reaction mixture was the same as given in 1), except that dried cell suspension (1.4 mg. N.) was used as an enzyme preparation instead of cell-free extract.

5) During incubation, pyruvate and fumarate were formed from OAA and malate, respectively, by the enzymic action of the extract (*1*).

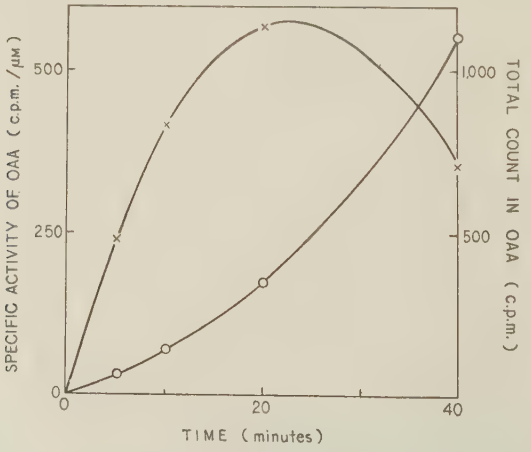

FIG. 2. Incorporation of C^{14} from $C^{14}O_2$ into oxaloacetate by cell-free extract (—○—, specific activity; —×—, total count). Conditions were the same as given in 1) of Table III.

tion of TPN spectrophotometrically (*1*). The CO_2-malate exchange reaction

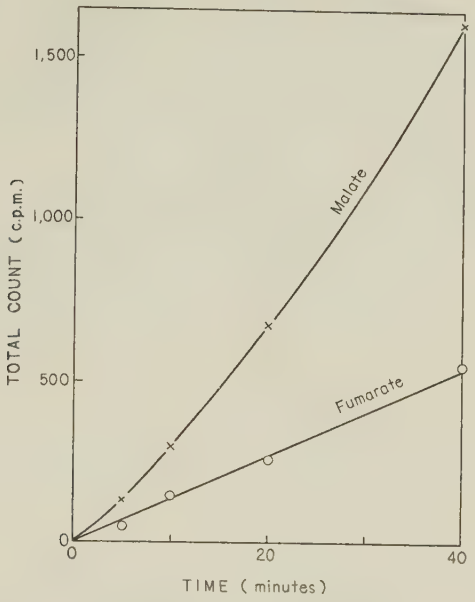

FIG. 3. Incorporation of C^{14} from $C^{14}O_2$ into malate by cell-free extract.

Conditions were the same as given in 2) of Table III.

TABLE IV

Effect of Cofactors on the C^{14}-Incorporation into Malate and Oxaloacetate from Carbon Dioxide

Substrate	Incubation mixture	C^{14}-Incorporation			
		Total count		Specific activity	
		cpm.	%	cpm./μM	%
Malate	Complete system (I)	1,310[1)]	100		
	No TPN	882[1)]	67		
	No $MnSO_4$	207[1)]	16		
	No malate	15[1)]	1		
Oxaloacetate	Complete system (II)	620		200	100
	No ITP	349		129	65
	No $MnSO_4$	534		121	61
	No oxaloacetate	0		—	—

1) Sum of counts in malate and fumarate.

Complete system (I) and (II) were essentially the same as shown in 2) and 1) of Table III, respectively. Cell-free extract containing 0.318 mg. N. (dialysed against 0.01 *M* Tris buffer, pH 7.5, for 2 hours) was used as enzyme. Incubated at 37° for 20 minutes.

shown in Table III and Fig. 3, and its dependence on the presence of TPN and manganous ions can be explained by the presence of "malic" enzyme.

The results of Table IV indicate that two separate carboxylation reactions may be operative, the one by OAA carboxylase and the other by "malic" enzyme. Hence it seemed necessary to elucidate the relationship

TABLE V

The Relationship between Oxaloacetate and L-Malate in Carbon Dioxide Fixation by Cell-free Extract

Experiment	Incubation mixture	Specific Radioactivity	
		Oxaloacetate	L-Malate
		cpm./μM	
1	Mixture A	137	7.5
	Mixture B	44.3	3.4
2	Mixture B	39.4	3.3
	Mixture C	—	64.7

Mixture A: complete system II of Table IV plus 40 μM of malate.
Mixture B: complete system I of Table IV plus 80 μM of OAA.
Mixture C: complete system I of Table IV.

Enzyme preparations used were dialysed cell-free extract containing 0.53 mg. N. in Exp. 1 and undialysed cell-free extract containing 0.44 mg. N. in Exp. 2. Incubated at 37° for 20 minutes.

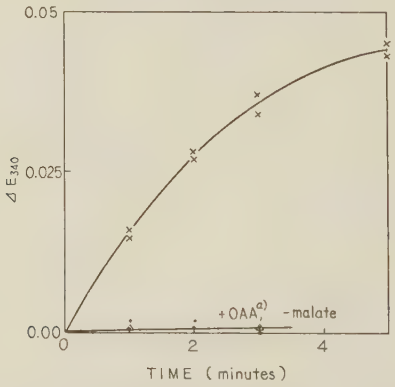

FIG. 4. Effect of oxaloacetate on the TPN reduction with malate by cell-free extract.

Reaction mixture contained 10 μM of potassium L-malate, 0.125μM of TPN, 0.5μM of $MnSO_4$, 50μM of Tris buffer, pH 7.5, and cell-free extract containing 0.22 mg. N. in a final volume of 1 ml. at 20°.

a) 10 μM of OAA (K-salt) was added.

between these two reactions by examining under various conditions the relative specific activity of OAA and malic acid produced. If malate is a precursor of OAA in the CO_2-OAA reaction, the specific activity of the former substrate would not exceed that of the latter throughout the reaction. As shown in Table V, the specific activity of OAA is more than 18 times as high as that of the malate, making it unlikely that the latter is a precursor in this reaction. Table V also shows that the CO_2-malate exchange reaction is inhibited strongly by the addition of OAA. Likewise, TPN reduction measured spectrophotometrically is suppressed by the addition of OAA in the system where the activity of malic enzyme was otherwise observed (Fig. 4). These situations made it difficult to obtain further evidence that the fixation of CO_2 into malate with the help of TPN did not involve OAA as precursor in this case.

DISCUSSION

The presence of an enzyme system in *B. flavum*, which forms glutamic acid from acetic acid was reported in the previous paper (*19*). This system,

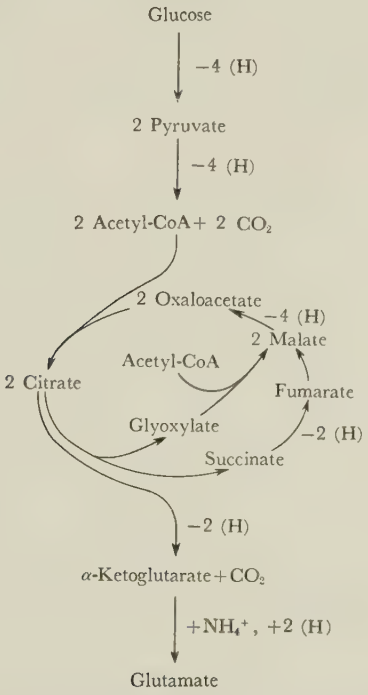

FIG. 5. Glutamate formation from glucose through the modified Krebs cycle with the glyoxylate bypass.

a modification of the Krebs cycle, can also explain the accumulation of glutamic acid from glucose and some other materials (Fig. 5). However, the

β-carboxylation of pyruvic acid (or PEP) and succeeding reaction in the Krebs cycle seem to provide an alternative pathway for the formation of glutamic acid from glucose (Fig. 1).

The present study showed that glutamic acid formed was labelled almost exclusively in the α-carboxyl carbon when glucose and C^{14}-bicarbonate were incubated aerobically with whole cell of this organism. Labelling of the α-carboxyl carbon could be explained simply by assuming the occurrence of the isotope exchange reactions that might take place between four carbon dicarboxylic acids of the Krebs cycle, three carbon units and radioactive carbon dioxide, as is the case with glutamate formation from acetate (*19*). But this is considered unlikely for the following reasons. First, specific activity of glutamate or α-ketoglutarate derived from glucose in the presence of C^{14}-bicarbonate is far greater than that derived from acetate, oxaloacetate, and succinate, and no CO_2 fixation reaction is involved in the pathways of glutamic and α-ketoglutaric acid formation from these acids as shown in Fig. 1 or 5. Secondary, specific activity of glutamic or α-ketoglutaric acid is greater than that of CO_2 isolated after the reaction only when glucose is used as substrate and this would be expected if the net fixation of CO_2 occurs to a considerable extent. Therefore, it is suggested that in the glutamic acid formation from glucose the pathway given in Fig. 1 may also operate, for which CO_2 fixation to form dicarboxylic acid of the Krebs cycle is considered as a key reaction.

In the case of pyruvate as substrate, specific activities of the products are similar to those derived from acetate rather than from glucose. This means that in this case main pathway may be the one illustrated in Fig. 5.

It is possible that the small amount of the isotope found in C-2 to C-5 of glutamate was introduced *via* pyruvate, which had been labelled in all three carbon atoms by the following process: $C^{14}O_2$ can be first introduced into the carboxyl group of pyruvate by way of a symmetrical dicarboxylic acid, *e.g.* fumaric or succinic. Incorporation of carboxyl-labelled pyruvate into hexose, and the subsequent metabolism of the latter through the pentose phosphate cycle, which occurs to a certain extent in this bacterium (*3*), would yield pyruvate labelled in all its carbon atoms.

The results with the cell-free extract of this bacterium showed that the cells contained the enzyme systems catalyzing the incorporation of C^{14} from $C^{14}O_2$ into OAA and L-malate. The incorporation into OAA required ITP and manganous ions while the incorporation into malate required TPN and manganous ions for full activity. From a study of the possible relationship of these two CO_2 fixing mechanisms in the cell extract by estimating the relative rates of incorpration of C^{14} into the pools of malate and OAA, it can be concluded that the incorporation of CO_2 into OAA does not involve malate as an intermediate. These results indicate the presence of OAA carboxylase of Utter and Kurahashi (*6-8*) in this bacterium. In 1959 the presence of this enzyme in heterotrophic bacteria was reported for the first time by Werkman *et al.* (*22*).

On the other hand, the requirements of TPN and manganous ions for

full activity and the spectrophotometric evidence for the presence of "malic" enzyme in this cells suggests that the incorporation of labelled CO_2 into malate is catalyzed also by "malic" enzyme, although none of the above results constitutes direct evidence for the hypothesis that OAA is not involved in the reaction as an intermediate.

Labelling of the α-carboxyl carbon atom of glutamic acid in the $C^{14}O_2$ fixation by whole cells of this organism can be explained by the participation of these β-carboxylating enzymes.

However, a remarkable difference of its specific activity observed between glucose and pyruvate as substrate would be ascribed to the preferential participation of OAA carboxylase in the whole cells, since it is rather widely held that the reversal of the pyruvate kinase reaction leading to net synthesis of PEP *in vivo* is insignificant (*23–26*).

SUMMARY

1. When each of glucose, acetate, pyruvate, oxaloacetate, and succinate was incubated aerobically with the washed cell suspension of *Brevibacterium flavum* No. 2247 in the presence of $C^{14}O_2$, labelled glutamate and α-ketoglutarate were obtained in all cases. The specific activities of these acids were far greater with glucose than that with other substrates. The carbon atom of the α-carboxyl group of glutamate thus formed had a specific activity about 100 times as great as the average of that found in the other carbon atoms.

2. With the cell-free extract of this bacterium incorporation of a considerable amount of C^{14} from $C^{14}O_2$ into oxaloacetate and L-malate was observed. The incorporation into oxaloacetate was stimulated by the addition of inosine triphosphate and manganous ion, and that into L-malate by the addition of triphosphopyridine nucleotide and manganous ion, suggesting both the oxaloacetic carboxylase of Utter and Kurahashi and the malic enzyme of Ochoa to be involved in the $C^{14}O_2$ fixation.

3. On the basis of the relative rates of incorporation of C^{14} into pools of malate and oxaloacetate, it was concluded that the incorporation of CO_2 into oxalocetate did not involve malate as intermediate.

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STUDIES ON THE OXIDATIVE DECOMPOSITION OF UROCANIC ACID*

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Since Sera (*1*) first demonstrated that urocanic acid is decomposed by a liver enzyme, many investigations have been made on the metabolism of urocanic acid. The conversion of urocanic acid to glutamic acid via formamidinoglutaric acid has been elucidated recently.

Evidence is now accumulating that imidazolonepropionic acid, a proposed early intermediate in urocanic acid metabolism, is readily hydrolyzed and oxidized (*2*, *3*).

The authors previously demonstrated that urocanic acid was also oxidatively decomposed. Succinic monoureid, hydantoinacrylic acid, α-ketoglutaric acid amide and other oxidation products were isolated by the authors (*4*, *5*), Suda *et al.* (*6*) and Uchida *et al.* (*7*). Recently, Brown and Kies (*8*) showed the formation of hydantoinpropionic acid *in vitro* and *in vivo*. Miller and Wealsch (*9*) reported the reduction of 2,6-dichlorophenolindophenol (CPIP) in the enzymatic decomposition of urocanic acid. The authors have partially purified urocanase from *Pseudomonas aeruginosa* and from cat liver using CPIP reduction as a measure of urocanase activity, and found that the oxidation arises from imidazolonepropionic acid and requires H_2O_2 in both enzyme preparations.

EXPERIMENTALS

Materials and Methods

1. Acetone powder of *Pseudomonas aeruginosa*—*Pseudomonas aeruginosa* cells were inoculated into 20 liters of neutral broth and cultured in a jar-fermenter for 18 hours at 35°, with aeration. The flow of air was 20 liters per minute. Cells were harvested in a Sharples centrifuge and were suspended in 20 volumes of a medium containing histidine (0.1 per cent L-histidine; 0.5 per cent NaCl; 0.1 per cent K_2HPO_4; 0.1 per cent $(NH_4)_2HPO_4$; 0.04 per cent $MgSO_4 \cdot 7H_2O$, pH 7.4) and incubated for 3 hours at 35°, with aeration.

* This work was presented, in part, at the 10th Symposium on Enzyme Chemistry (Japan) in 1958, at the Kinki Local Meeting of Japanese Biochemical Society in May 1959, and at the 32nd Annual Meeting of Japanese Biochemical Society in November 1959.

The adapted cells were again collected with a Sharples centrifuge and washed with physiological saline. Acetone treatment of the cells yielded about 40 g. of powder. The acetone-dried cells did not lose urocanase activity significantly on storage at −5° for about 6 months.

2. Beef liver catalase was prepared by the method of Sarkar and Sumner (*10*), and assayed with $KMnO_4$ by Bonnichsen's rapid titration method (*11*). The Katalasefähigkeit of the preparation used was about 12,000.

3. Urocanic acid and urocanic acid-2-C^{14} were prepared enzymatically from L-histidine and L-histidine-2-C^{14} respectively according to the procedure of Mehler, Tabor and Hayaishi (*12*) with a partial modification. Some of the L-histidine-2-C^{14} was kindly given to us by Dr. Richard W. Shayer. To isolate and purify urocanic acid-2-C^{14} of high specific activity, the incubation mixture was deproteinized with trichloroacetic acid, filtered and placed on a column of Amberlite IR 112, H-form. After washing with a sufficient amount of water to remove anionic impurities, adsorbed urocanic acid was eluted with 1.5 *N* NH_4OH. The eluate was then passed through the column of Amberlite IRC 50, H-form. All the cationic impurites were retained on the column. The filtrate* which contained urocanic acid-2-C^{14} was evaporated to dryness *in vacuo*, and a slightly colored powder was obtained, which was identified as urocanic acid-2-C^{14} by paper chromatography and by paper electrophoresis**.

4. DEAE cellulose was made from cellulose powder (cotton) (Toyo Roshi) by the method of Peterson and Sober (*14*).

5. The urocanase activity was assayed by one of the following two methods.

(i) By the reduction of CPIP, *i. e.* following the decrease in optical density of CPIP at 600 mμ in a thermojacketed Shimadzu QB 50 spectrophotometer. The urocanase preparation, 0.3 μM of CPIP and 100 μM of phosphate buffer, pH 7.4, in a total volume of 2.9 ml, were preincubated at 35°. After 10 minutes, 1.0 μM (0.1 ml.) of urocanic acid was added and the initial rate of decrease in optical density of CPIP was recorded. The reduction of CPIP obeyed zero order reaction until it was 30 per cent reduced. The amount of enzyme which caused a decrease in optical density of 0.10 per minute was defined as a unit of urocanase activity. The validity of this assay system will be discussed later.

(ii) By following the decrease in optical density at 277 mμ of urocanic acid, as described by Tabor and Mehler (*15*).

6. Oxygen uptake was determined in a Warburg manometer.

7. Protein was determined by measuring the turbidity at 340 mμ after addition of trichloroacetic acid (*16*).

8. α-Ketoglutaric acid amide was prepared enzymatically. α-Ketoglutaric acid amide was determined as its 2,4-dinitrophenylhydrazone. To a sample containing less than 0.5 μM of α-ketoglutaric acid amide, 0.1 ml. of 0.5 per cent 2,4-dinitrophenylhydrazine in 2*N* HCl was added. After incubation for 30 minutes at 37°, the mixture was extracted with 2.0 ml. of ethyl acetate. To the ethyl acetate layer, 4.0 ml. of 20 per cent Na_2CO_3 were added, the mixture well shaken. The optical density of sodium carbonate layer was measured spectrophotometrically at 378 mμ.

9. Urea and other carbamyl compounds were detected by the method of Koritz and Cohen (*17*).

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* As we usually used a sufficient amount of histidine deaminase, L-histidine-2-C^{14} did not remain in the incubation mixture. But, if necessary, it can be removed simply by IRC 50 resin buffered at pH 4.7 with ammonium acetate-acetic acid (*13*).

** It is readily recrystallized from water in the presence of a small amount of carriers.

10. Synthetic formamidinoglutaric acid was a gift of Dr. Heinrich Wealsch. Formamidinoglutaric acid was determined with alkaline ferricyanide-nitroprusside reagent, as described by Rabinowitz and Pricer (*18*). Formamidinoglutaric acid was also detected on paper chromatograms by spraying them with the same reagent.

11. Paper chromatography and paper electrophoresis—Ascending paper chromatography in two different solvent systems and paper electrophoresis were employed for the separation and identification of urocanic acid and some of its degradation products. Solvent I consisted of *n*-butanol, glacial acetic acid, water (4 : 1 : 1). Solvent II consisted of *n*-propanol, 0.2 *N* NH_4OH (3 : 1). Paper electrophoresis was carried out at high voltages (*e. g.* 5.5 KV for 40 minutes) in a buffer system of glacial acetic acid, pyridine, water (10 : 1 : 90), pH 3.6 (*19*). Toyo Roshi No. 51-A filter papers of 40 cm. length were used for paper chromatography, and 55 cm. length for paper electrophoresis. Urocanic acid, hydantoinacrylic acid, α-ketoglutaric acid amide and α-ketoglutaric acid were detected on papers by spraying them with diazotized sulfanilic acid and 4 per cent NaOH. To detect formylisoglutamine, a 0.5 per cent solution of ninhydrin in butanol was sprayed on the paper, which was then thoroughly heated for at least 10 minutes. Hydantoinpropionic acid was detected on paper chromatograms by spraying them with brom-phenol-blue solution or a freshly prepared mixture of $AgNO_3$ and NH_4OH solutions (1 : 1). A white spot appeared against a brown background in the position of hydantoinpropionic acid (*8*). Carbamylglutamic acid and urea were detected on papers by spraying them with 2 per cent *p*-dimethylaminobenzaldehyde in 20 per cent HCl. Carbamylglutamic acid, hydantoinpropionic acid and hydantoinacrylic acid were synthesized according to the procedure described by Dakin (*20*). Formylisoglutamine was prepared enzymatically. The R_f values of the compounds in Solvents I and II are summerized in Table V.

12. Radioactivity was determined by a gas flow counter (Nuclear Chicago).

RESULTS AND DISCUSSION

Purification of Bacterial Urocanase—Twenty g. of acetone-dried cells of *Pseudomonas aeruginosa* were ground with 40 g. of sea sand and extracted with 600 ml. of distilled water for two hours. After centrifugation at 12,000 × *g* for 20 minutes, the residue was again extracted with 100 ml. of water, and centrifuged. The combined supernatants were used as a crude extract. The urocanase activity remained in the supernatant after centrifugation at 140,000 × *g* for 1 hour. To the crude extract, 5 per cent streptomycin sulfate was added to a final concentration of 0.2 per cent. After standing for 30 minutes, the mixture was centrifuged at 12,000 × *g* for 15 minutes and the residue discarded. The streptomycin-treated supernatant was then heated for 5 minutes at 60° in a water bath and after rapid cooling, recentrifuged.

To the supernatant obtained, were added 20.9 g. of solid ammonium sulfate per 100 ml. of enzyme solution (0.35 saturation). After centrifugation, 16.4 g. of solid ammonium sulfate per 100 ml. of the supernatant solution were added (0.6 saturation). After 2 hours, the mixture was centrifuged at 12,000 × *g* for 20 minutes. The precipitate was dissolved in 30 ml. of 0.05 *M* phosphate buffer, pH 7.4 and dialyzed overnight (about 14 hours) against running water at about 10°.

The dialyzed fraction was adjusted to pH 5.0 with 5 per cent acetic acid, the precipitate which had formed was removed, and the pH was raised

to 5.6. The supernatant was treated with DEAE cellulose 10 times greater in weight than that of protein in solution. The cellulose used was previously buffered with 0.01 *M* phosphate buffer, pH 5.6. The cellulose was washed twice with 60 ml. of 0.01 *M* phosphate buffer, pH 5.6, and then the protein was eluted with 10 aliquots (15 ml. each) of Na_2HPO_4 solution ranging from 0.02 *M* to 0.2 *M*. The fractions which showed high urocanase activity were combined.

The combined eluates were fractionated with neutralized saturated ammonium sulfate and the fraction precipitating between 0.5 and 0.62 saturation was disolved in 30 ml. of 0.05 *M* phosphate buffer, pH 7.4 (Fraction AS II). A summary of the purification procedure is presented in Table I.

TABLE I

Purification of Urocanase from Pseudomonas aeruginosa

Procedure	Total volume	Protein	Specific activity	Total units	Recovery
	ml.	mg.	units/mg. protein		%
Extract	600	3900	0.31	1209	100
Streptomycin	600	1800	0.63	1134	94.4
Heat treatment	570	598.5	1.80	1077.3	89.1
1st $(NH_4)_2SO_4$	30	130.5	8.00	1044	86.3
DEAE cellulose	100	68	13.7	931.6	77.1
2nd $(NH_4)_2SO_4$	30	32.4	21.9	709.6	58.7

Purification of Urocanase from Cat Liver—One hundred and fifty g. of fresh cat liver were ground with an equal amount of sea sand and the mixture extracted with 300 ml. of distilled water for 2 hours. The coarse matter was removed by centrifugation. The extract was adjusted to pH 5.0 with 5 per cent acetic acid, and the precipitate which formed was removed by centrifugation. The pH of the supernatant was raised to about 7 with 4 per cent NaOH. Centrifugation at 12,000×*g* yielded 210 ml. of slightly turbid red supernatant. 17.4 g. of solid ammonium sulfate were added per 100 ml. of supernatant and after 1 hour the mixture centrifuged at 12,000×*g* for 20 minutes. The supernatant solution was again treated with 19.8 g. of solid ammonium sulfate per 100 ml. of solution and the mixture centrifuged at the same speed as above (0.3–0.6 saturation). The precipitate was dissolved in 140 ml. of 0.01 *M* phosphate buffer, pH 7.4 (Fraction AS I). A portion of 24.3 g. of solid ammonium sulfate were added per 100 ml. of this solution, and the resulting precipitate was dissolved in 140 ml. of 0.01 *M* phosphate buffer, pH 7.4 (Fraction AS II). This fraction was then mixed with calcium phosphate gel (gel/protein=1.0) at neutral pH. After 15 minutes the gel was removed by brief centrifugation (Fraction CG). To the supernatant solution, urocanic acid was added to a final concentration of 0.04 *M* (*6*) and the mixture heated for 1 minute at 63°. The denatured protein was centri-

fuged off, and the supernatant was treated with 4 volumes of saturated ammonium sulfate solution to remove urocanic acid (Fraction H). Heating of Fraction CG in the absence of added urocanic acid resulted in complete loss of urocanase activity.

Attempts to purify the enzyme by calcium phosphate gel adsorption or by alcohol or acetone fractionation were unsuccessful.

The purification procedure is summarized in Table II.

TABLE II

Purification of Urocanase from Cat Liver

Procedure	Total volume	Protein	Specific activity	Total units	Recovery
	ml.	mg.	units/mg. protein		%
Extract	276	8644.3	0.64	5566.9	100
Acid pptn	210	3666.6	0.77	2834.3	50.9
1st $(NH_4)_2SO_4$	143	2486.7	1.11	2770.3	49.8
2nd $(NH_4)_2SO_4$	140	904.4	2.28	2064.8	37.1
$Ca_3(PO_4)_2$ gel	143	491.9	3.79	1863.4	33.5
Heat treatment	109	133.0	4.67	621.3	11.2

TABLE III

Effect of Inhibitors on Urocanase Activity

0.05 mg. of bacterial Fraction AS II and 0.06 mg. of liver Fraction H were used.

Urocanase activity was determined by CPIP reduction; conditions were as described in "Materials and Methods".

Inhibitor	Concentration	Per cent inhibition	
		Bacteria	Liver
	M		
Hydroxylamine[1]	1×10^{-4}	68.9	0
Bisulfite[1]	1×10^{-4}	83.3	71.1
Hydrosulfite[1]	1×10^{-4}	81.2	69.7
Semicarbazide	1×10^{-3}	0	0
Azide	1×10^{-3}	0	0
KCN	5×10^{-3}	84.0	88.3
	1×10^{-3}	40.0	30.0
Nitroso-R	1×10^{-3}	37.0	64.3
R-acid	1×10^{-3}	0	0
PCMB[2]	5×10^{-5}	25.0	76.2

1) Urocanase activity was determined by the decrease in optical density of urocanic acid at 277 mμ. Bisulfite and hydrosulfite caused nonenzymatic reduction of CPIP.

2) *p*-Chloromercuribenzoate.

Some Properties of Bacterial and Liver Urocanase—The pH dependence of urocanase activity was studied by using CPIP reduction as a measure of urocanase activity, as described above. The pH optimum in both enzyme preparations lay between 7.0 and 8.0. There was low activity in borate or Tris (hydroxymethyl) aminomethane buffer than in phosphate or pyrophosphate buffer.

Both bacterial and liver urocanase were inhibited by preincubation with various reducing agents. Table III shows the effect of inhibitors on urocanase activity. Activity was determined either by the rate of CPIP reduction or by the decrease in optical density at 277 mμ. No appreciable inhibition was observed by preincubation with 1×10^{-3} *M* EDTA, α,α—dipyridyl or oxine.

Imidazolonepropionic Acid as the Starting Substrate for the Oxidation—When the liver enzyme fractions before the heat treatment (Fractions AS I, AS II, and CG) were incubated with urocanic acid, no oxygen uptake was observed and the end product was formamidinoglutaric acid. Oxygen consumption

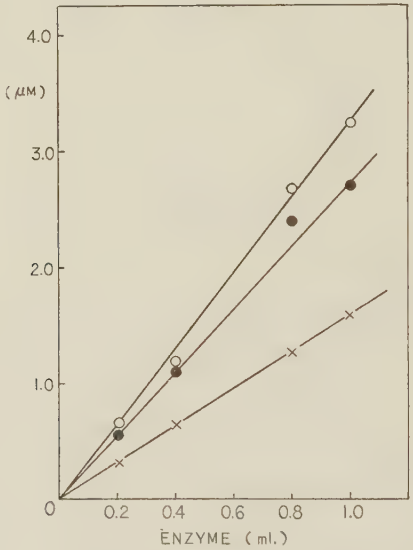

FIG. 1. UCA* disappearance, O_2 uptake and KGAA formation as a function of enzyme concentration.

Incubation mixture contained: 100 μM of phosphate buffer, pH 7.4, 6 μM of UCA, and Fraction H protein as indicated, in a total volume of 3.0 ml. The mixture was incubated in a Warburg vessel for 120 minutes at 37°.
—○— UCA disappeared, —●— O_2 consumed
—×— KGAA formed

* The following abbreviations are used: UCA=urocanic acid, KGAA=α-ketoglutaric acid amide, FAG=formamidinoglutaric acid, FIG=formyl-DL-isoglutamine, HPA=hydantoinpropionic acid, HAA=hydantoinacrylic acid.

Table IV

Conversion of UCA to FAG and KGAA by Various Enzyme Preparations from Cat Liver

Preparation	UCA disappeared	FAG formed	KGAA formed
	μM	μM	μM
Fraction AS I	1.00	1.00	0.00
Fraction AS II	1.00	1.00	0.00
Fraction CG	1.00	1.00	0.00
Heat denaturation			
at 55°	1.00	0.71	0.00
at 60°	1.00	0.10	0.48
at 63°	1.00	0.07	0.46

Conditions: 150 μM of phosphate buffer, pH 7.4, 6 μM of UCA, and 0.6 ml. of enzyme solution, in a total volume of 2.9 ml. were incubated for 60 minutes at 37°. The reaction was stopped by the addition of 0.1 ml. of 60% perchloric acid. 0.5 ml. aliquots were taken for each determination.

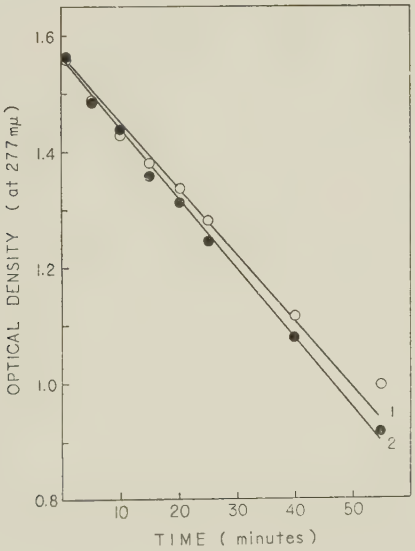

Fig. 2. The rate of UCA disappearance under aerobic and anaerobic conditions.

Incubation mixture contained: 100 μM of phosphate buffer, pH 7.4, 0.2 μM of UCA, and 0.24 mg. of Fraction H protein in a total of 3.1 ml. The mixture was incubated in Thunberg-type cuvettes, at room temperature (about 20°).

1. anaerobic 2. aerobic,

and formation of α-ketoglutaric acid amide and substances which gave a positive carbamyl reaction after treatment with alkali for 10 minutes at 100°, were observed when Fraction H from cat liver or the enzyme from *Pseudomonas aeruginosa* were used. The formation of α-ketoglutaric acid amide, which is known to be one of the oxidation products of urocanic acid (*6*), O_2 uptake and urocanic acid disappearance were correlated as shown in Fig. 1. As shown in Table IV, α-ketoglutaric acid amide but no formamidinoglutaric acid were formed from urocanic acid by the heat treated enzyme preparation. It is generally accepted that urocanic acid is converted to formamidinoglutaric acid through imidazolonepropionic acid, and it is reasonable to assume that heat treatment may destroy an enzyme or enzymes in this pathway, and that urocanic acid or the intermediate accumulating (imidazolonepropionic acid) may be oxidized.

Fig. 2 shows the rate of urocanic acid disappearance under aerobic or anaerobic conditions. The rate of urocanic acid decomposition was the same under both conditions. If oxidation were to occur at the level of urocanic acid, the rate of urocanic acid disappearance under aerobic conditions would be greater than that under anaerobic conditions. This would indicate that the intermediate, imidazolonepropionic acid, is oxidized.

Because of its lability, attempts to isolate or synthesize imidazolonepropionic acid were unsuccessful. Recently, it is reported that imidazolonepropionic acid is readily hydrolyzed non-enzymatically forming formyl-DL-isoglutamine (*2*, *3*). Therefore, using the enzyme preparation which does not form formamidinoglutaric acid form urocanic acid (Fraction H), it was anticipated that, anaerobically, the end product will be formylisoglutamine. This was substantiated by isotopic experiments using urocanic acid-2-C^{14}. When urocanic acid-2-C^{14} was incubated with Fractions AS I, AS II and CG, all the radioactivity was found in a spot corresponding to formamidinoglutaric acid. Formamidinoglutaric acid formation was almost completely suppressed by incubation with Fraction H. The radioactivity chiefly accumulated in a spot corresponding to formylisoglutamine, especially in the absence of oxygen. As discussed above, this supports the idea that heat treatment destroys the imidazolone hydrase of Feinberg and Greenberg and the imidazolonepropionic acid accumulating is converted non-enzymatically to formylisoglutamine.

Under aerobic conditions, incubation of urocanic acid-2-C^{14} with Fraction H resulted in new radioactive spots on a paper chromatogram and decreased activity in formylisoglutamine. Only 58.6 per cent of the radioactivity incubated could be accounted for from the chromatograms. That is, 41.4 per cent of the radioactivity was lost during the incubation or in subsequent procedures. Therefore, the formation of some volatile material was suspected. In addition to the formation of α-ketoglutaric acid amide, these results are thought to be due to formation of oxidation products of the imidazolonepropionic acid which accumulated.

Miller and Wealsch (*9*) reported that when urocanic acid was incubated with cat liver urocanase, dyes such as CPIP or ferricyanide were

reduced. Feinberg and Greenberg (*3*) have shown that ferricyanide was reduced non-enzymatically by imidazolonepropionic acid. We have shown that the reduction of CPIP also occurs non-enzymatically. Urocanic acid was incubated with the enzyme under N_2, and the incubation mixture was heated at 100° for 1 minute to destroy the enzyme. The reduction of CPIP by this heated incubation mixture is shown in Fig. 3. Formamidinoglutaric acid, formylisoglutamine and urocanic acid added to heat-inactivated

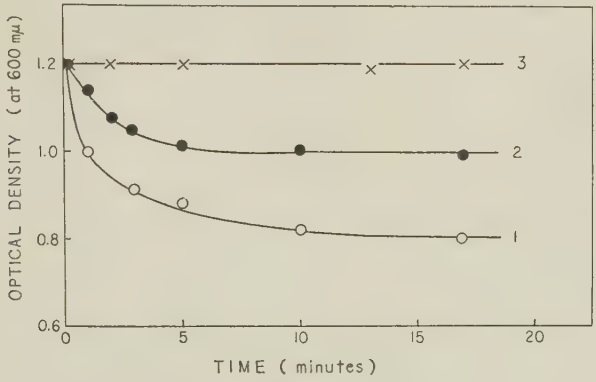

FIG. 3. Non-enzymatic reduction of CPIP by a heated incubation mixture.

100 μM of phosphate buffer, pH 7.4, 2 μM of UCA, and 0.24 mg. of Fraction H protein were incubated for 60 minutes at 37°, anaerobically. The incubation mixture was then placed in boiling water for 1 minute. A 0.5 ml. aliquot was added to a cuvette containing 0.25 μM of CPIP in 2.5 ml.

1. Sample taken directly after incubation, 2. Sample stored overnight in a refrigerator, 3. Heated enzyme plus UCA.

enzyme caused no reduction of dye. This experiment proves the validity of the assay procedure of urocanase using CPIP reduction. To oxidize imidazolonepropionic acid, an excess amount of CPIP or an H_2O_2-generating system together with horse-radish peroxidase, were added to the incubation mixture. The enzyme fraction forming formamidinoglutaric acid from urocanic acid and causing no oxygen uptake was employed. The reaction products in this case were identical with those observed using Fraction H, *i.e.* α-ketoglutaric acid amide and the substances giving a positive carbamyl reaction after alkali-treatment. Miller and Wealsch (*9*) isolated α-ketoglutaric acid by treating the incubation mixture containing CPIP with HCl. It is suggested that the α-ketoglutaric acid amide formed under these conditions was hydrolyzed to α-ketoglutaric acid by HCl.

From the above results it is concluded that the oxidation arises from the common intermediate, imidazolonepropionic acid, which is readily hydrolyzed and oxidized.

The Fate of the 2-C^{14} of the Imidazole Ring in the Decomposition of Urocanic

Acid-2-C^{14}—As mentioned above, there are two ways in which urocanic acid-2-C^{14} can be oxidized. One is represented by some spots detected on paper

Table V

R_f Values of Variout Compounds

Compound	R_f Value	
	Solvent I[1)]	Solvent I[2)]
UCA	0.56	0.31
Histidine	0.11	0.19
FIG	0.47	0.22
FAG	0.17	0.09
HPA	0.55	0.22
HAA	0.61	0.26
Carbamylglutamic acid	0.51	0.09
Urea	0.50	0.46
KGAA	0.60	—

1) Solvent I: *n*-butanol, glacial acetic acid, water (4 : 1 : 1).
2) Solvent II: *n*-propanol, 0.2 *N* NH_4OH (3 : 1).

chromatograms which appear only when oxygen was present. Another is some volatile materials which are detected by chromatography from a loss in total counts. It seemed possible that the volatile compound formed from the 2-C^{14} of the imidazole ring might be formic acid or carbon dioxide. Dilution assays were carried out with carrier formic acid and carbon dioxide to determine which was formed. The incubation mixture was acidified with sulfuric acid, quickly frozen with Dry Ice-acetone, and lyophilized. The volatile radioactivity was trapped by dilute NaOH, and this solution was divided into two parts. To one half, HCOONa was added as a carrier, the formate was recrystallized from a water-ethanol system. After a second recrystallization, crystals with a constant specific activity were obtained. To the other half, a sufficient amount of Na_2CO_3 was added as a carrier followed by the addition of $Ba(OH)_2$ solution, and the $BaCO_3$ precipitating was washed repeatedly with hot water, centrifugation being used to recover the precipitate. The radioactive material obtained on lyophilization of the incubation mixture, which was thought to be formic acid, contained the expected radioactivity, *i.e.* all the missing counts could be accounted for in the formic acid. Conversely, essentially no radioactivity was found in the $BaCO_3$.

The radioactivity in the formic acid always accounted for 40–45 per cent of that in the urocanic acid-2-C^{14} decomposed while, under the same conditions, α-ketoglutaric acid amide and free ammonia formation also accounted for 40-45 per cent of the urocanic acid decomposed. Free ammonia was determined by the microdiffusion method of Conway using saturated potassium carbonate. It is reasonable to assume that about 45 per cent of

the urocanic acid decomposed is converted to α-ketoglutaric acid amide, formic acid and ammonia. These form one group of oxidation products.

TABLE VI

Distribution of Reaction Products under Various Conditions

Enzyme fraction		% of incubated radioactivity found on paper chromatogram				
		Total radioactivity found on paper	UCA decomposed	FAG formed	FIG formed	Others formed
AS II	Aerobic	100	100	100	0	0
	Anaerobic	100	100	100	0	0
H	Aerobic	58.6	100	0	6.9[1]	51.7[1]
	Anaerobic	100	100	0	75.2	24.8

Reaction mixture contained 10 μg of UCA-2-C^{14} (0.16 μc.), 0.56 mg. of Fraction AS II protein or 0.28 mg. of Fraction H protein, and 10 μM of phosphate buffer, pH 7.4 in a total volume of 0.2 ml. The mixture was incubated for 30 minutes in the presence or absence of O_2. 0.3 ml. of hot ethanol was added to the incubation mixture, and it was dried *in vacuo* (overnight), 0.8 ml. of water was added and 0.1 ml. aliquots were applied quantitatively to filter paper. After chromatography, the paper was cut into 1/4 inch wide strips, which were counted directly. To identify formylisoglutamine and formamidinoglutaric acid, three combinations of co-chromatography (paper chomatography in two solvent systems and paper electrophoresis) were performed for a sample. The respective carriers were mixed with the samples before application to paper. Formamidinoglutaric acid and formylisoglutamine were determined by their radioactivity which coincided with the color developed by the respective carriers. When the radioactivity of the compounds overlapped, corrections were made by comparison with the papers chromatographed in other systems. As a control, a reaction mixture before incubation was co-chromatographed in the same manner. This indicated the total radioactivity incubated.

1) Actual percentage of radioactivity found on paper was 10.1 for FIG, and 89.9 for other compounds. Corrections were made from the loss of radioactivity on paper.

Besides α-ketoglutaric acid amide and formic acid, the formation of some carbamyl derivatives was suspected because hydantoinacrylic acid (*5*), hydantoinpropionic acid (*8*) and succinic monoureid (*4*) had previously been isolated from the reaction mixture. We have shown the occurrence of hydantoinpropionic acid-C^{14} in the urine of a normal rat after intraperitoneal injection of urocanic acid-2-C^{14}. This confirms the view of Brown and Kies (*8*). Unexpectedly, the direct occurrence of hydantoinacrylic acid, hydantoinpropionic acid and carbamylglutamic acid after incubation of urocanic acid-2-C^{14} with a cat liver enzyme were not observed by successive

co-chromatography on paper*.

Recently, in this laboratory, an unknown product with the formula of $C_6H_8N_2O_5$, was isolated from an incubation mixture containing the pseudomonad enzyme. The compound gave a carbamyl reaction. This compound yielded hydantoinacrylic acid on treatment with strong HCl, and urea and α-ketoglutaric acid on treatment with NaOH (these compounds were detected by paper chromatography). This was thought to be ureidoglutaconic acid, but as the authentic material could not be obtained, its chemical nature is still unknown.

The procedure used for the isolation of this compound was as follows. A reaction mixture containing 5 g. of urocanic acid and enzyme obtained from 40 g. of an acetone powder of *Pseudomonas aeruginosa*, in a total volume of 3 liters, was adjusted to pH 7.4 with NaOH and incubated at 37° with shaking. After urocanic acid had been completely decomposed, the incubation mixture was deproteinized with perchloric acid. The excess perchloric acid was removed with $KHCO_3$. The solution was evaporated to 40 ml. *in vacuo*, and desalted with ethanol. One hundred and twenty ml. of Dowex 50, H-form (100–200 mesh) were added to the concentrate, stirred and removed by filtration. The filtrate was then chromatographed on a 2.5×30 cm. column of Dowex 1, Cl-form (120–200 mesh). On eluting with 0.02 *N* HCl, 500 mg. of $C_6H_8N_2O_5$ and 100 mg. of α-ketoglutaric acid amide were obtained from separate fractions. One hundred mg. of α-ketoglutaric acid was then eluted from the column with 0.06 *N* HCl.

It was shown more clearly in an isotopic experiment that there is some precursor of hydantoinacrylic acid in the incubation mixture. After carrier hydantoinacrylic acid had been added to the incubation mixture, the mixture was boiled with 6 *N* HCl (final concentration) for 60 minutes. Trapping of the radioactivity in hydantoinacrylic acid was confirmed by successive co-chromatography on paper*.

On paper chromatography of an aerobic incubation mixture, two major spots of unknown compounds were observed. Their radioactivity is converted to urea after hydrolysis with NaOH. This was shown after eluting these two spots from the paper separately. Urea was added to each eluate as carrier, and the mixtures were then hydrolyzed with 2 *N* NaOH. The

* A reaction mixture containing 15 μM of phosphate buffer, pH 7.4, 15 μg. of urocanic acid-2-C^{14} (ca. 0.25 μc.) and 0.42 mg. of liver Fraction H protein, in a total volume of 0.3 ml. was incubated for 30 minutes at 37°. 1 mg. of carrier and then 0.7 ml. of hot ethanol were added to the incubation mixture, and the mixture was dried *in vacuo*. Successive co-chromatography was carried out as following. All the sample were applied to filter paper as 6 cm. streaks and subjected to electrophoresis. A portion of the chromatogram was cut off, counted and color developed by spraying. The corresponding area was eluted from the remainder of the chromatogram which had not been sprayed. The eluate was again applied to paper, and the same procedure was repeated in Solvent I and II successively. At each step, radioactivity and color development were checked. Finally, multiple development in Solvent I or II was used to test if the compounds had separated from each other.

products were identified by successive co-chromatography*. One of these two compounds might be the precursor of the hydantoinacrylic acid mentioned above.

We now have a direct evidence, in addition to α-ketoglutaric acid amide formation, that oxidation occurs at the C-2 position of the imidazole ring. This may result in some unknown carbamyl derivatives or their precursor which are not identical with hydantoinacrylic acid, hydantoinpropionic acid or carbamylglutamic acid. The nature of these compounds and their relationship to α-ketoglutaric acid amide formation is now under investigation.

Role of Hydrogen Peroxide and Consideration of the Mechanism of Oxidative Decomposition of Urocanic Acid—The oxygen consumption during urocanic acid decomposition is gradually reduced when the unit of catalase added was increasing. This also resulted in the prolongation of the lag period before oxidation occurred in both bacterial and liver enzyme preparations. With sufficient amount of catalase oxygen uptake was finally completely inhibited (Figs. 4 and 5). This complete inhibition is interesting and suggests that

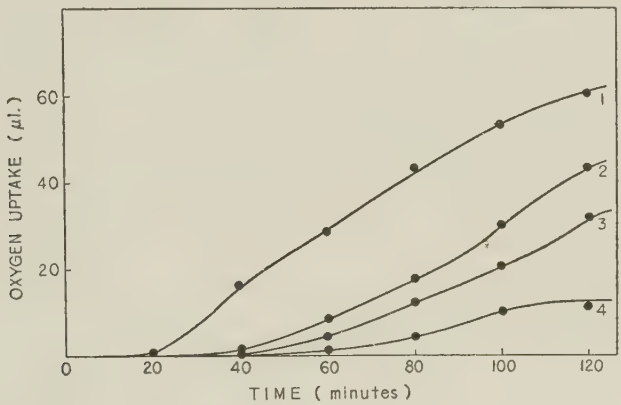

Fig. 4. Effect of catalase on oxygen uptake during UCA decomposition by a liver enzyme.

Incubation mixture contained, in a total volume of 3.0 ml., 100 μM of phosphate buffer, pH 7.4, 6 μM of UCA, 6.0 mg. of liver Fraction H protein and catalase preparation.

Curve 1) No added catalase, 2) 0.90 mg. of catalase, 3) 1.80 mg. of catalase, 4) 3.60 mg. of catalase.

the oxidative reaction involves hydrogen peroxide, and at least in the first step, differs from non-enzymatic or flavin-linked oxidation reaction. A dependency on hydrogen peroxide was also observed in the following experiments. The lag period in oxygen uptake was eliminated by the addition of a small amount of hydrogen peroxide (0.5 μM) (Fig. 6). The elimination of the lag in oxygen uptake by addition of hydrogen peroxide was not noted

* See the footnote of p. 132.

using the bacterial enzyme which contained catalase as a contamination. Moreover, 5×10^{-4} *M* of Mn^{++} could eliminate the requirement for hydrogen peroxide in preventing the lag period. An analogous metal activation was

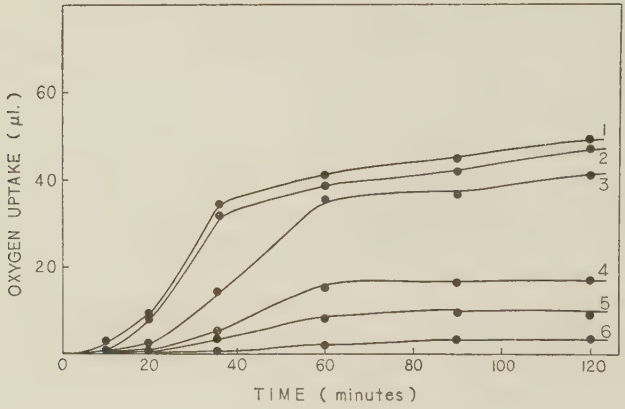

Fig. 5. Effect of catalase on oxygen uptake during UCA decomposition by a bacterial enzyme.

Incubation mixture contained, in a total volume of 3.0 ml., 100 μM of phosphate buffer, pH 7.4, 6 μM of UCA, 0.43 mg. of bacterial Fraction AS II protein and catalase preparation.

Curve 1) No added catalase, 2) 0.09 mg. of catalase, 3) 0.18 mg. of catalase, 4) 0.46 mg. of catalase, 5) 0.92 mg. of catalase, 6) 1.84 mg. of catalase.

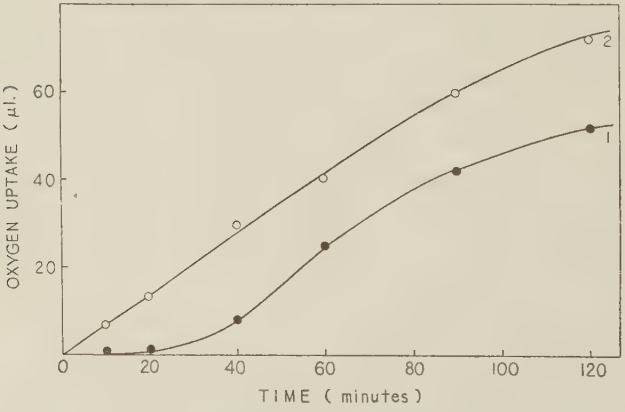

Fig. 6. Elimination of the lag period in the oxidation by hydrogen peroxide.

Incubation mixture contained, in a total volume of 3.0 ml. 100 μM of phosphate buffer, pH 7.4, 6 μM of UCA, 6.0 mg. of liver Fraction H protein. Incubation at 37°. In Curve 2, 0.5 μM of H_2O_2 was added.

observed with the bacterial enzyme. Diethyldithiocarbamate treated enzyme caused no oxygen uptake, but on incubation with Cu^{++}, its oxygen uptake

was recovered. Other metals tested, such as Co^{++}, Ni^{++}, Zn^{++}, Fe^{++} and Fe^{+++}, were without effect. Versene ($1\times10^{-3}\,M$) also partially inhibited the oxidation (Fig. 7).

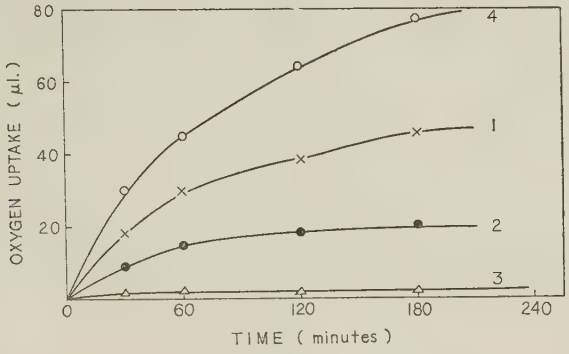

Fig. 7. Inhibition of oxygen uptake during UCA decomposition by some metal-chelators and the reveral of inhibition by Cu^{++}.

Incubation mixture contained, in a total volume of 3.0 ml., 100 μM of phosphate buffer, pH 7.4, 5 μM of UCA, and 0.3 mg. of bacterial Fraction AS II protein. Incubation at 37°.

Curve 1) no addition, 2) $1\times10^{-3}M$ of Versene added, 3) Fraction AS II, which had been preincubated with diethyldithiocarbamate for 30 minutes at 4°, and dialyzed against water for 6 hours at 4°, was used. The urocanase activity was not diminished by this treatment, 4) $1\times10^{-5}\,M$ of Cu^{++} was added to the same system as that of Curve 3.

It is uncertain whether the metal acts as a catalyst in non-enzymatic oxidation of the oxidizable substrate (imidazolonepropionic acid) thus providing hydrogen peroxide for the first step of the oxidation or it is an activator of the oxidizing enzyme.

The requirement for hydrogen peroxide and the subsequent oxygen uptake raises the problem about it operating mechanism. One possibility is the oxidase action of peroxidase, the substrates of which are such as dihydroxyfumaric acid (*21*, *22*), indoleacetic acid (*23*, *24*), and triose reductone (*25*). Hydrogen peroxide is used as a reactant, and successive or by-standing oxidation occurs forming more hydrogen peroxide. The hydrogen peroxide thus formed is utilized in the reaction. In such systems, an activation by Mn^{++} has been reported. It is interesting that the substrate of system such as these are easily oxidizable, and the activation by Mn^{++} has been reported. They are very similar to our oxidation system.

Another possibility is a case such that with tryptophan pyrrolase (*26*, *27*). In this reaction hydrogen peroxide is used as an activator of the enzyme, and hydrogen peroxide is not consumed in oxidation of the substrate. Both the above reactions are catalyzed by a porphyrin enzyme and inhibited by KCN.

Urocanase which offers the substrate of the oxidation, imidazolonepropionic acid, is inhibited by KCN. Therefore, the effect of KCN on the urocanate oxidizing enzyme system is not clear. Furthermore, the oxidation of urocanate was not inhibited by CO (CO 20 per cent N_2 80 per cent), although tryptophan pyrrolase is. Azide was also not inhibitory.

The activation by metal ions, and ready oxidizability of imidazolonepropionic acid suggest that the enzyme concerned with urocanic acid oxidation may be closely related to the enzymes of the first group discussed above. The substrate of this oxidation, imidazolonepropionic acid, is so labile that it can not be isolated.

However, an attempt to demonstrate peroxidase activity of the enzyme used using reduced indophenol dye or guaiacol as a hydrogen donor was unsuccessful. Therefore, elucidation of the true mechanism of the oxidation must await further investigation.

SUMMARY

1. Urocanase was partially purified from *Pseudomonas aeruginosa* and from cat liver, measuring urocanase activity by CPIP reduction.

2. The pH optimum of the urocanase was found to lie between pH 7.0 and 8.0 in both enzyme preparations. Effects of some inhibitors on urocanase were studied.

3. Imidazolonepropionic acid, a proposed early intermediate in urocanate metabolism, is thought to be the starting substrate for the oxidation.

4. The oxidation was completely inhibited by catalase, and stimulated by some metals. A lag period in the oxidation was eliminated by the addition of a small amount of hydrogen peroxide. The mechanism of the oxidation is discussed.

5. One group of oxidation products of urocanic acid includes α-ketoglutaric acid amide, formic acid and ammonia. Another is represented by some carbamyl derivatives or their precursor. The occurrence of these compounds in the incubation mixture was demonstrated using urocanic acid-2-C^{14}.

Before this paper was submitted to press, papers of Brown and Kies have appeared (*28*). They claim that hydantoinpropionic acid arises from imidazolonepropionic acid.

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STUDIES ON TAKA-MALTASE

II. SUBSTRATE SPECIFICITY OF TAKA-MALTASE I

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Many investigators (*1*) studied so for in detail the substrate specificity of yeast α-glucosidase which hydrolyses maltose, its derivatives and α-glucosides, and Weidenhagen (*2*) proposed a general theory on the carbohydrase specificity. However, contradictory results were also reported on the substrate specificity of maltase in Taka-diastase. Some (*3*, *4*) reported that Taka-maltase hydrolysed maltose but not α-glucosides, while others (*5*, *6*) reported that hydrolysis of α-glucosides by this enzyme was at a lower rate than that of maltose. Since the maltose-hydrolysing activity of Taka-diastase is derived from saccharogenic amylase (amyloglucosidase) (*7*) and α-glucosidase, the reported substrate specificity of Taka-maltase must be based on the study of a combined activity of these two carbohydrases and consequently there remains considerable ambiguity. Therefore, a new study must be conducted on purified α-glucosidase in order to clarify the substrate specificity of Taka-malatse.

The previous paper (*8*) dealt with the isolation of Taka-maltase from the discarded rivanol supernatant in the crystallization course of Taka-amylase A from Taka-diastase, and the present paper will report the studies on the substrate specificity using various α-glucosides and α-maltosides.

METHODS AND MATERIALS

Enzyme—Taka-maltase I was purified according to the procedure described in the previous paper (*8*). The enzyme solution thus prepared contained 0.089 mg. of total nitrogen per ml.

Substrate—The following crystalline compounds were synthesized as indicated: α-methyl-D-glucoside (*9*), α-phenyl-D-glucoside (*10*), α-methyl-maltoside (*11*) and α-phenyl-maltoside (*12*, *13*). Other compounds were purchased as follows: trehalose from Difco Laboratories, raffinose from Schering-Kahlbaum A.G., amylose from Nagase Co. Ltd., sucrose from Wako Junyaku Co. Ltd. Lactose and cellobiose were prepared in this laboratory.

Analytical Procedure—One volume of ten-folds diluted enzyme solution, one volume of 0.1 *M* substrate solution, four volumes of 0.5 *M* acetate buffer solution of pH 4.7 and four volumes of water were mixed and kept at 30°. Aliquot portions of the reaction mixture were pipetted out for analysis at appropriate intervals and liberated glucose was

estimated by Somogyi-Nelson's colorimetry (*14*, *15*) and liberated phenol by Folin's phenol reagent (*16*). The rates of hydrolysis were indicated by the decomposition percentage of the substrates.

Test on the Transglucosylation—One ml. of enzyme solution, 5 ml. of 2 per cent maltose solution and 4 ml. of water were mixed and kept at 30°. Samples, which were withdrawn at intervals from the reaction mixture, were heated at 100° for five minutes to inactivate the enzyme, condensed in a vacuum desiccator and applied to the paper chromatography in the ascending way, repeating the development three times on the same filter paper of "Toyo No. 51" using the solvent mixture of *n*-butanol : pyridine : water (6 : 4 : 3 by volume) and aniline-hydrogenphthalate was used for detection of sugar spots.

The examination on the transglucosylation was conducted on the hydrolysates of α-glucosides or α-maltosides at the 24th hour in the above mentioned analytical procedure through the paper chromatography after inactivation by heating at 100° for five minutes and desalting by Dowex-50 (H^+ form), and spots of sugars were detected by the same reagent.

RESULTS

The rates of hydrolysis obtained by the enzymatic action at pH 4.7 on the substrates of 0.01 *M* are shown on the Table I and in Fig. 1. The rela-

Table I

Rates of Hydrolysis of Maltose, α-Methyl-D-Glucoside, α-Phenyl-D-Glucoside, α-Methyl-Maltoside and α-Phenyl-Maltoside by Taka-Maltase I

The same values are plotted in Fig. 1.

Time hr.	Per cent hydrolysis of substrate						
	Maltose	α-Methyl-glucoside	α-Phenyl-glucoside		α-Methyl-maltoside	α-Phenyl-maltoside	
			Glucose	Phenol		Glucose	Phenol
1/6	27.8	—	—	—	0.4	15.3	0.8
1/2	52.1	0.4	3.2	7.7	0.8	25.7	4.0
1	62.7	0.8	6.9	15.7	1.6	31.3	9.2
2	76.7	1.5	13.9	27.3	2.5	39.6	18.4
4	86.7	2.2	26.4	46.9	3.9	50.7	32.8
6	91.5	2.9	37.1	64.5	4.9	—	45.4
8	—	4.0	47.9	75.8	6.0	60.4	55.0
24	93.9	8.5	92.3	100.0	11.4	90.3	99.7

tive rates of hydrolysis were maltose : α-methyl-D-glucoside : α-phenyl-D-glucoside = 1 : 0.0048 : 0.092 and maltose : α-methyl-maltoside : α-phenyl-maltoside = 1 : 0.0096 : 0.54. In the present experiment it was shown that Taka-maltase I did not hydrolyse amylose, sucrose, raffinose, trehalose, cellobiose and lactose.

As shown in Fig. 2, several oligosaccharides were produced by the transglucosylation. Therefore, Taka-maltase I was proved to have the activity of transglucosylase. Chromatographic studies indicated that panose and

isomaltose were produced primarily at the initial stage of reaction, and isomaltotriose and trace of higher oligosaccharides were produced later, as

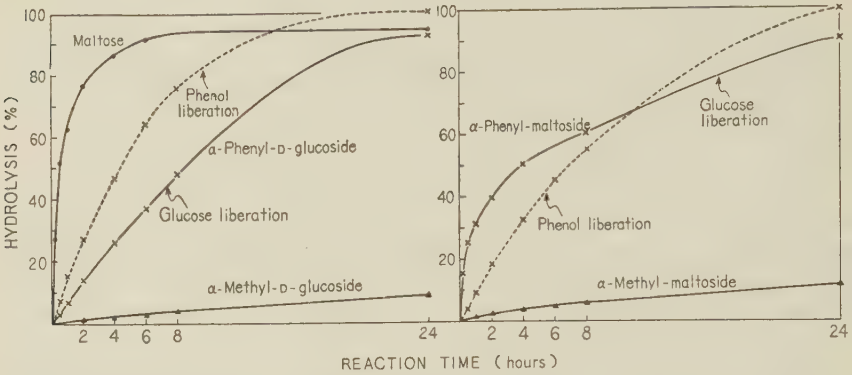

Fig. 1. Time curves of enzymatic hydrolysis of maltose, α-methyl-D-glucoside, α-phenyl-D-glucoside, α-methyl-maltoside and α-phenyl-maltoside by Taka-maltase I.

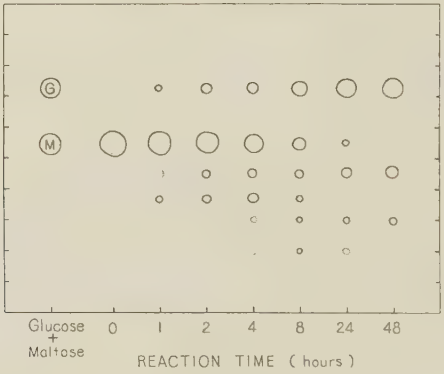

Fig. 2. Paper chromatogram of the transglucosylation products of maltose by Taka-maltase I.
Solvent; *n*-butanol : pyridine : water (6 : 4 : 3)

Pazur (*17*) reported. With advanced of reaction, panose was decomposed and at the final stage isomaltose and isomaltotriose were main products of the transglucosylation.

Twenty four hours action of Taka-maltase I on α-methyl-D-glucoside, α-phenyl-D-glucoside and α-phenyl-maltoside led to the formation of isomaltose.

Changes in optical rotatory power were observed during the hydrolytic action of Taka-maltase I on maltose. The accelerated mutarotation of glucose so far liberated, after the enzymatic action was stopped by the addition of an excess of alkali at various times, was measured. The results are shown on the Table II. The data show that the mutarotation of glucose liberated from maltose by the action of Taka-maltase I is downward. The evidence indicates, when

maltose is hydrolysed to glucose, α-glucose is liberated without change in the configuration.

TABLE II

Changes in Optical Rotation of the Reaction Mixture during the Hydrolytic Action of Taka-Maltase I on Maltose at 30°

Time min.	Observed optical rotation		
	During hydrolysis α_D	After the addition of alkali α'_D	$\alpha'_D - \alpha_D$
0	2.63	—	—
10	2.51	2.16	−0.35
30	2.43	2.00	−0.43
60	2.08	1.86	−0.22
120	1.88	1.78	−0.10

Substrate concentration 0.025 *M*; enzyme solution 5 ml.; 0.1 *M* acetate buffer of pH 4.7; total volume 40 ml.

DISCUSSION

In the present experiment, Taka-maltase I is proved to have three enzymatic activities. The first is the maltose hydrolysing holosidase activity. The second is heterosidase activity, hydrolysing α-phenyl-D-glucoside readily and α-methyl-D-glucoside at much lower rates, in analogy with Weidenhagen's experiment on Taka-diastase (*5*) and Sugawara and others' experiment on maltase from *Aspergillus oryzae* (*18*). The third is transglucosylase activity, by which oligosaccharides are formed by transfer of α-glucosyl residue to the substrate or products as Pazur (*16*) and Miwa (*19*) have previously pointed out.

In comparing the substrate specificity of Taka-maltase I with that of α-glucosidase in yeast, the relative rate of hydrolysis of Taka-maltase I on maltose, α-methyl-D-glucoside and α-phenyl-D-glucoside is 1 : 0.0048 : 0.092 while that of α-glucosidase in yeast (*20*) is 1 : 0.43 : 5.2. That is, Taka-maltase I hydrolyses maltose more rapidly than α-glucosides, and conversely α-glucosidase in yeast hydrolyses α-phenyl-D-glucoside more rapidly than maltose. α-Glucosidase in yeast hydrolyses sucrose, though Taka-maltase I does not. Thus the substrate specificity of Taka-maltase I is far different from that of α-glucosidase in yeast.

The rate of hydrolysis of α-phenyl-maltoside by Taka-maltase I is about one-half of that of maltose. The experimental data show that the liberation of glucose at the initial stage of the hydrolysis distinctly exceeds the liberation of phenol. The fact shows that α-1,4-glucosidic linkage between two glucose residues in α-phenyl-maltoside is split at first resulting in the formation of glucose and α-phenyl-D-glucoside and the latter of the products is split further

into glucose and phenol. The rate of hydrolysis of α-methyl-maltoside is about one-hundredth of that of maltose, and only glucose, but no maltose, is detected paper chromatographically in the hydrolysate. Judging from the mode of splitting of α-phenyl-maltoside, it must be reasonable to consider that the splitting of α-methyl-maltoside also takes place at the α-1,4-glucosidic linkage between two glucose resitues and not at the glucosidic linkage between methyl residue and maltose residue. Though both α-methyl-maltoside and α-phenyl-maltoside belong to α-maltosides, the rates of hydrolysis of glucosidic linkage between two glucose residues in both α-maltosides are far different from each other according to the nature of aglycon residue. These facts might be explained by the characteristics of chemical structure of the aglycon groups.

Leibowitz (*21*) classified maltases into two different types. One type is glucosido-maltase represented by α-glucosidase in yeast and small intestine which can split both maltose and all α-glucosides and has affinity to α-glucosyl residue. The other type is gluco-maltase represented by maltase from mold and barely malt which can hydrolyse maltose only and has affinity to the reducing moiety of maltose. However, Taka-diastase contains, besides maltase, Taka-amylase B (amyloglucosidase) which is capable of hydrolysing starch and maltose (*7*). So, it is quite probable that Leibowitz dealt with only one of these two enzymes in mold, namely amyloglucosidase only, in his experiment. In the present studies, it is clearly demonstrated that Taka-maltase I belongs to α-glucosidase capable of hydrolysing not only maltose but also α-methyl-D-glucoside and α-phenyl-D-glucoside. Therefore, if Leibowitz's theory is generally valid, Taka-maltase I must belong to glucosido-maltase. Considering from the difference in relative activities of Taka-maltase I upon maltose, α-methyl-maltoside and α-phenyl-maltoside, it is reasonable to assume that Taka-maltase I has affinities not only to α-glucosyl residue but also to reducing moiety of maltose. The fact that the activity of Taka-maltase I remarkably decreases when the reducing group of maltose is masked could be explained by the above assumption.

Ben-Gershom (*22*), based upon his experiment where upward mutarotation was observed during action of Taka-diastase on maltose or cyclohexaglucan, reported that Taka-maltase and cyclohexaglucanase in Taka-diastase brought about anomeric inversion of configuration of hydrolysate in analogy with the inversion observed with β-amylase. However, in the present experiment it is observed that in the action of purified Taka-maltase I on maltose no anomeric inversion occurs, contrary to Ben-Gershom's observation.

Although it is not obvious whether or not Taka-amylase B (amyloglucosidase in Taka-diastase) brings about the change in configuration at hydrolysis of maltose, upward mutarotation is reported to be observed during the action of γ-amylase (amyloglucosidase in *Aspergillus awamori sp.*) on dextrin (*23*). Ben-Gershom's observation of upward mutarotation during the action of maltase in Taka-diastase on maltose, is supposed to be caused by the maltase activity of amyloglucosidase, but not by the action of α-glucosidase.

SUMMARY

The substrate specificity of purified Taka-maltase I was studied. The relative rates of hydrolysis of Taka-maltase I on maltose, α-methyl-D-glucoside, α-phenyl-D-glucoside, α-methyl-maltoside and α-phenyl-maltoside were 1, 0.0048, 0.092 0.0096 and 0.54, respectively. Amylose, sucrose, raffinose, trehalose, cellobiose and lactose were not attacked by this enzyme.

Taka-maltase I also showed the transglucosylase activity. Downward mutarotation of glucose produced by hydrolysis of maltose by this enzyme was observed.

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BIOCHEMICAL STUDIES ON THE FRESH-WATER MEDUSA CRASPEDACUSTA SOWERBYI LANKESTER

II. DISTRIBUTION OF THIAMINE RIBOFLAVIN AND FREE AMINO ACIDS*

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In the preceding paper an attempt has been made to the inorganic composition of fresh-water medusa *Craspedacusta sowerbyi* (*1*). This Hydrozoa belongs to phylogenetically primitive animal and the lowest grade of the tissue-levels in histological differentiation, the body being consisted of two layers of cells, between which is found a noncellular substance, the mesogrea, as of gelatin, which constitutes the bulk of the body. Probably this may be hydro-medusa grown up within two or three months from under 1 mm. to 18 mm. in diameter and in adult, four gonads have developed, which are consisted simply of germcells aggregated in them. This is rare material as fresh water Coelenterata, whose distribution is restricted in the world and of which little biochemical studies have been made. This note described that the results obtained from the first part of the study, chiefly the distribution of thiamine, riboflavin and free amino acids in the fresh-water medusa *C. sowerbyi* in comparison with that of marine medusa *A. aurita*.

The fresh-water medusae (*C. sowerbyi*)were collected at Itamiike in the vicinity of Itami-City, Hyogo Prefecture, at the end of August, 1957. The marine medusae (*A. aurita*) were obtained from Suma Coast of Osaka Bay on the near date to collected *C. sowerbyi*.

For the quantitative analysis of thiamine, and riboflavin, the desiccated materials were used. Thiamine, (V. B_1) was estimated by Fujita's thiochrome fluorometric method (*2*), and riboflavin, (V. B_2) by the lumiflavin method devised by the same author (*3*). The amounts of V. B_1 and of V. B_2 in fresh weight 100 *g* of *C. sowerbyi* and *A. aurita* are shown in Table I.

Free amino acids were separated by paper chromatography, and their presence in the paper was proved by the ninhydrin reaction. The procedure depended on the following methods; the fresh medusae were ground in Potter-Elvehjem glass homogenizer with a 10-fold amount of 85 per cent ethanol. The ethanol precipitate was removed by centrifugation, the supernatant was,

* A part of this work was read at the annual meeting of the Zoological Society of Japan, held at Matuyama in October, 1958.

after being concentrated *in vacuo*, centrifuged. This concentrated solution was used for chromatographic sample. Ten to forty microliters of the sample

TABLE I

The Quantity of Thiamine and Riboflavin in the Medusae, C. sowerbyi and A. aurita

Animal	Fresh materials	Thiamine		Riboflavin
		Total	Free	
C. sowerbyi	100 g.	406.4 μg.	335.7 μg.	1166.0 μg.
A. aurita	100	331.0	297.7	144.7

were applied on filter paper, Toyo Roshi No. 51, and were run with a solvent mixture, *n*-butanol : glacial acetic acid : water (4 : 1 : 2 v/v) or saturated phenol at room temperature. The chromatograms were dried after running for 13–15 hrs., the spots were derived with spraying of 2 per cent ninhydrin solution and then heated for a few minutes at 90–100°. Nine free amino acids, cystine, aspartic acid, glutamic acid, taurine, glycine, threonine, alanine, β-alanine and phenylalanine were detected.

These amino acids were identified by their characteristic positions and colors on the resultant chromatograms, comparing them with the trial runs of authentic samples and also with the R_f values published by Satake and others (*5*, *6*). But it could not be separated of free amino acids in *A. aurita* for unknown obstacles. From the result of only these free amino acids in *C. sowerbyi* we could not describe the significant difference to the related animals. In the studies of free amino acids in Protozoa by Warren Lee and others (*7*, *8*)reported that differences in amino acid metabolism may be related to difference in morphology of the related species. It is an interesting problem, but at present time I have no other data, accordingly it must be refered to further studies.

The chemical analysis gave the results that *C. sowerbyi* and *A. aurita* respectively contained water 98.98 and 87.68 per cent, total solids 1.02 and

TABLE II

The Quantity of Protein, Fat and Carbohydrate in 100 g. of Fresh Medusae, C. sowerbyi and A. aurita

Animal	Total organic substances	Crude proteins	Crude fats	Carbohydrates	Ashes
C. sowerbyi	0.927 g.	0.562 g.	0.089 g.	0.267 g.	0.098 g.
A. aurita	0.215	0.141	0.004	0.070	2.100

2.32 per cent, crude proteins 0.562 and 0.140 per cent, crude fats 0.089 and

0.004 per cent, carbohydrates 0.267 and 0.070 per cent and ashes 0.098 and 2.100 per cent. The protein was caluculated from the amounts of total nitrogen which was estimated by Kjeldahl's macro-method (*4*), the crude fats obtained by Soxhlet apparatus, and the carbohydrates calculated from the quantity of the total organic substances, proteins and fats. These results are shown in Table II.

C. sowerbyi contained the high concentration of thiamine, especially it contains a large amount of riboflavin, in comparison with that of marine medusa. The proportions of V. B_1 and V. B_2 in *C. sowerbyi* and *A. aurita.* were respectively 1 : 2.9 and 2.3 : 1 and total V. B_1 : free state V. B_1 was 1 : 0.82 and 1 : 0.89.

In the fresh-water medusa, the amounts of organic substances such as crude proteins, carbohydrates and crude fats were found to be 4 times, and 22 times respectively containing with those in marine medusa.

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OXIDATION OF ANTHRANILIC ACID CATALYSED BY PSEUDOMONAS CELL-FREE EXTRACT

By earlier investigations (*1–8*) it was shown in certain strains of *Pseudomonas* that anthranilic acid derived from tryptophan *via* kynurenine is further oxidised to form pyrocatechol, *cis-cis* muconic acid, and β-ketoadipic acid, successively. Though the formation of *cis-cis* muconic acid from pyrocatechol was successfully confirmed in extracts of acetone-dried cells and pyrocatechase, the responsible phenolytic oxygenase, was purified and shown to require ferrous ion (*4*, *5*, *8*), the active principle participating, in cell-free system, in the oxidation of anthranilic acid to pyrocatechol has not been demonstrated.

Preliminary studies revealed that a strain of *Pseudomonas aeruginosa* adapted to anthranilic acid produced a potent anthranilate oxidation system and it could be extracted as a soluble fraction. The bacterial cells were grown in a neutral bouillon-peptone medium containing 0.1 per cent of anthranilic acid at 37° for 15 hours, with mechanical shaking. The collected cells which were washed three times with water, suspended in two volumes of *M*/5 TRIS buffer, pH 7.8, supplemented with glutathione, were sonically disrupted (10 kc., 100 w., for 10 min.), and the cell-free extract was subsequently centrifuged at $100{,}000 \times g$ for 60 minutes to obtain a supernatant fraction which was used as the enzyme system in following studies. Difficulties in the extraction of the system must have been due, at least partly, to the lability of the induced enzyme in living microorganisms. Such an unstable property of induced enzymes may be commonly observed and typically demonstrated in this case. Therefore, minor changes of the culture conditions, *e.g.* amount of the substrate added, age of the bacterial cells, often resulted in unexpectedly broad fluctuation of the enzyme activity of the cell-free preparation. Optical condition of bacterial cultivation, in its strict meaning, must await further studies but the conditions described above were practically suitable for following experiments.

The *Pseudomonas aeruginosa* adapted to anthranilic acid oxidised pyrocatechol so readily as it did anthranilic acid. On the other hand, oxidations of 3-hydroxyanthranilic acid, 5-hydroxyanthranilic acid, 3,4-dihydroxyanthranilic acid, *o*-aminophenol, *o*-, *m*-, *p*-hydroxybenzoic acids, and protocatechuic acid were not stimulated after the adaptation to anthranilic acid (cf. *1*). These were also the case with the cell-free extract. Four atoms of oxygen per moles of anthranilic acid and two atoms per mole of pyrocatechol were consumed. One mole of carbon dioxide was evolved when anthranilic acid was decomposed. These results confirm the previous observation by several workers (*1*, *4–7*). Disappearance in anthranilic acid catalysed by the supernatant

was in good accordance with oxygen uptake. Simultaneous generation of ammonia was observed, though stoichiometric amount of ammonia was not determined, possibly because of employing crude enzyme preparation. After oxygen uptake had ceased, determination of β-keto acid was carried out by aniline-citrate method and it was found that β-ketoadipic acid was formed as one of the end products. Additions of lactate+DPN or glucose-6-phosphate +TPN were necessary for full activity at pH 7.4. The reaction was completely inhibited under anaerobic condition and severely in the presence of *o*-phenanthroline, diethyldithiocarbamate or *p*-chloromercuribenzoate, but was slightly or not affected by α,α'-dipyridyl, hydrogen peroxide or catalase. Complete inactivation of the preparation occurred after storage for three days in a refrigerator or in a freezer, showing its lability.

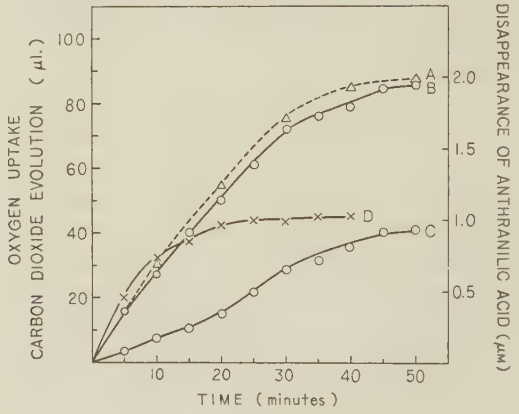

Fig. 1. Decompositions of anthranilic acid and pyrocatechol catalysed by *Pseudomonas* cell-free extract.

A: Disappearance of anthranilic acid.

B: Oxidation of anthranilic acid.

C: Evolution of carbon dioxide in the presence of anthranilic acid.

D: Oxidation of pyrocatechol.

Reaction mixture contained; Cell-free extract, 1.0 ml.; *M*/5 phosphate buffer (pH 7.4), 1.0 ml.; glucose-6-phosphate, 20 μmoles; TPN, 0.5 μmoles; anthranilic acid, 2 μmoles or pyrocatechol, 2 μmoles, total volume adjusted to 2.8 ml. with distilled water. Reactions at 37° under air.

These results suggest that (1) no stable intermediate between anthranilic acid and pyrocatechol would be formed, thus (2) only one enzyme in the presence of reduced pyridine nucleotides, which has (3) "oxygenase" properties, is concerned with and (4) heavy metals may participate in the reaction. Isolation and more precise characterisation of the enzyme are being carried out. Characterisation of the enzyme as an oxygenase will be achieved by introducing O^{18}. The O^{18} would be detected in two oxygen atoms in two

hydroxyl groups of pyrocatechol.

TABLE I

Requirement of Reduced Pyridinenucleotide Coenzymes For Anthranilic Acid Oxidation Catalysed by Pseudomonas Cell-Free Extract

Additions	Disappearance in anthranilic acid	Oxygen uptake
	(μmoles)	(μmoles)
None	0.03	—
Glucose-6-phosphate + TPN	1.19	2.3
Na-Lactate + DPN	1.65	3.1

Reaction mixture contained: Cell-free extract, 1.0 ml.; $M/5$ phosphate buffer (pH 7.4), 1.0 ml.; glucose-6-phosphate or Na-lactate, 20 μmoles; TPN or DPN, 0.5 μmoles; anthranilic acid, 3 μmoles, total volume adjusted to 2.8 ml.. with distilled water. Reactions for 15 minutes at 37° under air.

After the report was submitted to press, we found that Drs. Kuno and Akaishi (Dept. of Med. Chem., Med. School of Kyoto Univ., Kyoto, Japan) had succeeded in preparing an active enzyme preparation which carries out a conversion of anthranilic acid to pyrocatechol from anthranilate adapted cells of *Pseudomonas*. Their work was presented at the 4th Intern. Congr. Biochem., Vienna (1959), by Prof. O. Hayaishi.

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CALCIUM BINDING AND RELAXATION IN THE ACTOMYOSIN SYSTEM

Ebashi *et al.* (*1*) have reported studies comparing the relative abilities of some chelating agents to bind Ca and to cause relaxation of glycerinated muscle fibers. In these studies, no correlation between the two actions was found. The Ca binding data were taken from the paper of Danzuka and Ueno (*2*), the values representing binding of Ca by each chelating agent in the absence of any other divalent cation. The relaxation experiments, however, were carried out in 0.01 *M* Mg^{++}. It has now been found that, if the chelating activities for Ca are experimentally determined also in 0.01 *M* Mg^{++}, the ability to bind Ca correlates rather well with the relaxing action.

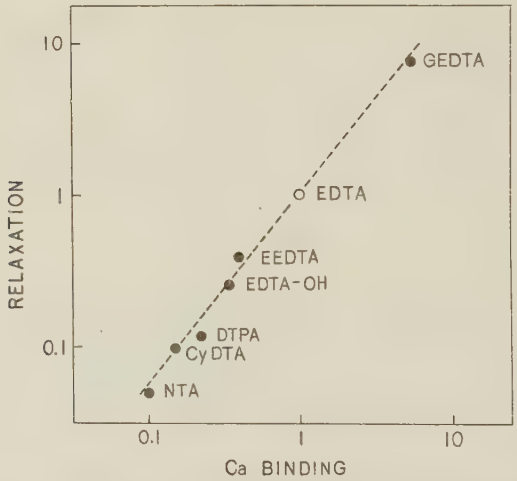

Fig. 1. Relationships between Ca binding and relaxing activities of chelating compounds.

Activity of each compound (●) was expressed as its value relative to that of EDTA (○) on the basis of molar concentration. Relaxing activities were quoted from the paper of Ebashi *et al.* (*1*). Details concerning the determination of Ca binding activity will be reported later. Abbreviations of compounds are the same as described in the paper of Ebashi *et al.* (*1*).

The abscissa of Fig. 1 shows the Ca binding activity of the compounds measured under the same conditions as the relaxing experiments, *i.e.* 0.15 *M* KCl and 0.01 *M* $MgCl_2$ at pH 6.8. The ordinate indicates the relaxing activity quoted from the paper by Ebashi *et al.* (*1*). Clearly, there is a linear relationship between the two actions.

Further experiments to be reported later in detail (*3*) show that the particulate fraction of skeletal muscle, which has relaxing activity (*4*), also strongly concentrates Ca in an ATP-coupled reaction and thereby removes it from the medium. Furthermore, work reported by Weber (*5*) and our own experiments indicate the role of Ca in the contraction of the actomyosin system. Therefore, since relaxing activity is correlated with Ca binding activity, and since Ca is required for contraction, it is proposed that removal of Ca has a direct connection with the onset of relaxation and that this may be the mechanism of the action of the natural particulate relaxing factor.

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PARTIAL COLLAGENASE DIGESTION OF THE FIBER STRUCTURE OF COLLAGEN

By the digestion with collagenase it was recently found (*1*, *2*, *3*) that a considerable portion of the collagen molecule has a structure of G-P-R type; G stands for a glycine residue, P for a proline residue and R for any other amino acid residue but mostly alanine or hydroxyproline. Only the G-P-R type structure is digested by collagenase (*1*). This kind of amino acid sequence has already been assumed (*4*) for the structure of collagen for the interpretation of X-ray diffraction pattern. When collagen was digested by collagenase, however, longer peptides of structure different from G-P-R type were also obtained (*3*).

The molecule of collagen is a rigid rod of 3000 Å in length (*5*, *6*, *7*, *8*), and it is generally accepted that such molecules associate with each other in various ways to form three kinds of collagen fibers (*9*). The segment long spacing (SLS) fibers (*10*) which are prepared by the addition of adenosinetriphosphoric acid (ATP) to the solution of collagen have uniform length of about 3000 A. It is assumed (*9*) that such fibers are formed by well aligned lateral association of collagen molecules. The SLS fibers show characteristic aperiodic striations running across the fiber, and the head, the tail and any other portion can readily be discerned on the electron micrograph.

In order to find out the location of the G-P-R type structure in a collagen molecule, SLS fibers were digested partially by collagenase and examined under the electron microscope.

The SLS fibers were prepared from rat tail collagen (Fig. 1), or from calf skin collagen (Fig. 2) by the addition of ATP to a final concentration of 0.1 per cent at pH 2.2. A drop of the suspension of SLS fibers was put on the plastic film, dried, and washed. A drop of collagenase in 0.05 *M* borate buffer solution of pH 7.4 was placed on the film with dry SLS fibers, and left to stand for a certain period of time in a closed chamber which was kept at a constant temperature of 20°. After the digestion reaction the specimen was washed, dried, and observed under the electron microscope.

The digestion of the fibers became visible on the electron micrograph after 20 minutes of collagenase treatment. In the initial stage, the digestion caused the elevated portions of the fiber to stand out more clearly, *i.e.* the parts in between the elevated striations were dissolved away (*cf.* Fig. 3). Then some of the striations were also attacked as the time of digestion passed. In the case of fibers from rat tail collagen, all the striations other than those particularly conspicuous striations near both ends of the fiber,

FIG. 1. SLS fibers from rat tail collagen (×37,000)

FIG. 2. SLS fibers from calf skin collagen. (×37,000)

FIG. 3. SLS fibers from calf skin collagen digested for 20 minutes with collagenase. (50,000)

FIG. 4. SLS fibers from rat tail collagen digested for 60 minutes with collagenase. (×37,000)

FIG. 5. SLS fibers from calf skin collagen digested for 60 minutes with collagenase. (×37,000)

dissolved at about the same rate, and at the end of a digestion period of 60 minutes, only the ends remained as is seen in Fig. 4. In the case of SLS fibers from calf skin, four thick striations, each corresponding to two or three fine striations were made stand out after 20 minutes of collagenase treatment (Fig. 3). Two of the four were at the ends of the fiber. One of the two thick striations located in the middle portion of the fiber was gradually dissolved, and after digestion for 60 minutes, only three remained (*cf.* Fig. 5). In the thick striation which remained after collagenase digestion, the fine structure of striations could not be identified.

A parallel experiment of the digestion of SLS fiber suspension with collagenase was also performed. From the measurement of the increase of free amino groups with ninhydrin colorimetry, the degree of digestion of collagen with collagenase was about 1/3 of full digestion after 60 minutes collagenase treatment. When the digestion product was roughly examined, it was found that the amount of tripeptides of G-P-R type increased roughly linearly with the time of collagenase treatment. However, it was very interesting to find that some non-dialysable peptide fraction remained and its amount reached a constant 40 minutes after the initiation of collagenase digestion.

Although it is still impossible to tell what part of the collagen molecule is made of the amino acid sequence of G-P-R, it was made certain by the present experiment that the amino acid structure of the collagen molecule is closely connected with the striations of the collagen fiber. A further detailed study of the amino acid structure of various portions of collagen molecules is now being carried on.

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ANTHRANILATE OXIDASE

By application of the concept of "successive adaptation" by Suda *et al.* (*1*) or "simultaneous adaptation" by Stanier (*2*), the metabolic pathway of tryptophan through anthranilic acid and catechol to β-ketoadipic acid had been established in bacteria. Most of the enzymes of this metabolic sequence were extracted in a cell-free state and their properties were studied in some detail. In spite of many efforts of various workers however, anthranilate oxidase, the enzyme catalyzing the conversion of anthranilate to catechol, has not been obtained in a cell-free state and consequently it has not been purified. Therefore the characteristics of this enzyme were entirely obscure.

We have succeeded in isolating anthranilate oxidase in a cell-free state from anthranilate-adapted cells, and have partially purified it.

The present communication is concerned with the extraction, partial purification and the mode of action of anthranilate oxidase.

The bacterium used in the experiments was isolated from garden soil. It is capable of growing aerobically with anthranilate as the source of both carbon and nitrogen. The bacterium was grown in a synthetic medium, containing anthranilate as the sole source of both carbon and nitrogen. It was harvested and stored at −20° until needed.

Ten grams of frozen cells were suspended in 20 ml. of egg albumin solution (containing 300 mg. of egg albumin) and subjected to sonic oscillation. Anthranilate oxidase can also be prepared by grinding the bacteria with alumina in the presence of egg albumin. The crude extract was further purified by protamin treatment and ammonium sulfate fractionation. Most of the experiments reported here were carried out with protamin-treated enzyme.

The highest anthranilate oxidase activity in crude extracts, was obtained around pH 8.0, and therefore further experiments were conducted at pH 8.0. However this pH is not necessarily the optimum for anthranilate oxidase, because our preparations are still contaminated with pyrocatechase.

Protamin-treated enzyme shows little activity towards anthranilate without supplementation with a TPNH-generating system (glucose-6-phosphate—glucose-6-phosphate dehydrogenase). A DPNH-generating system (D-lactic acid —lactic dehydrogenase) is a much less satisfactory substitute for the former system.

The reaction product of anthranilate oxidation was identified as β-ketoadipic acid by paper chromatography after conversion of the compound to its 2,4-dinitrophenylhydrazone.

From the stoichiometric experiments shown in Table I, it was demonstrated that the over-all reaction proceeded in the following molar ratio

disappearance of anthranilic acid : oxygen consumption : liberation of carbon dioxide : formation of β-ketoadipic acid, 1 : 2 : 1 : 1. It follows that two atoms of oxygen are needed for the conversion of anthranilic acid to catechol, because the latter compound was found to be metabolized to β-ketoadipic acid with consumption of two atoms of oxygen.

TABLE I

Stoichiometry of Enzymatic Degradation of Anthranilic Acid

Experiment No.	Anthranilate added	Anthranilate disappeared	Oxygen uptake	Carbon dioxide[1] evolved	β-Ketoadipate[2] formed
	μmoles	μmoles	μmoles	μmoles	μmoles
1	5.0	—	9.8	—	4.3
2	5.0	—	9.4	5.6	—
3	5.0	5.0[3]	—	—	—
4	5.0	4.4[4]	—	—	—

The reaction mixture contained; 0.4 ml. of enzyme (15 mg. of protein), 0.1 μmole of TPN, 2 μmoles of $MgCl_2$, 20 μmoles of glucose-6-phosphate, 0.05 ml. of glucose-6-phosphate dehydrogenase (100 μg.), and substrate as indicated. Tris-HCl buffer, pH 8.0, and egg albumin were added to make a final concentration of 0.1 *M* and 1 per cent respectively. The final volume was 2.0 ml. The reaction was carried out manometrically at 30° in air.

1) Calculated by the indirect Warburg method (*3*).
2) Determined by catalytic decrboxylation of 4-aminoantipyrine (*4*).
3) Determined by the modified method of Bratton and Marshall (*5*).
4) Estimated by decrease in absorption at 310 mμ (*6*).

Salicylic, 2,3-dihydroxybenzoic and benzoic acids, and *o*-aminophenol were all excluded as intermediates in anthranilate oxidation, because they were not attacked by the enzyme.

Azide and cyanide are potent inhibitors of anthranilate oxidase, the former showed 80 per cent inhibition at a concentration of 1×10^{-3} *M*, and the latter completely abolished the activity at a concentration of 2×10^{-4} *M*. On the other hand, aminopterin* has no inhibitory effect even at a concentration of 1×10^{-3} *M*. This result is inconsistent with those of Kaufman and Levenberg on phenylalanine hydroxylase (*7*).

Further purification of anthranilate oxidase can be efficiently achieved by ammonium sulfate fractionation, the enzyme being exclusively in the fraction precipitated at 0.33–0.50 saturation. It must be noted that the enzyme at this stage of purification exhibits no activity even in the presence of a TPNH-generating system unless reduced glutathione and a boiled extract of

* Aminopterin was kindly supplied by Dr. E. Ohmura, Takeda Research Laboratory, Osaka, Japan.

E. coli are added. Omission of any one of these three components completely abolished the enzyme activity. Ferrous ion has no effect.

Further purification of the enzyme and the reaction mechanism are now being investigated.

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ACTIVATION OF GLYCERATE 2,3-DIPHOSPHATASE BY MERCURIC ION

Glycerate 2,3-diphosphatase has been investigated by Rapoport *et al.* (*1*) and Joyce and Grisolia (*2*). Rapoport *et al.* prepared a crude enzyme preparation from rabbit muscle by ethanol fractionation, which was markedly activated by mercuric ion, whereas Joyce and Grisolia could not find this effect with a partially purified preparation obtained from chicken breast muscle. In order to clarify the reason of the discrepancy, following experiments were performed.

Rabbit muscle acetone powder was extracted with water and fractionated with ethanol according to Rapoport (*1*) (40–65 per cent fraction) (Preparation 1). This fraction was treated with mercuric acetate as described by Joyce and Grisolia. Excess of the metal was removed with IRC-50 and the enzyme was precipitated by addition of ethanol. The precipitate was dissolved in water and the clear supernatant was obtained by centrifugation (Preparation 2). At this step, the enzyme was purified about 40-fold compared with the original water extract. This preparation was dialyzed against cold 0.005 *M* s-collidine-HCl buffer at pH 7.0 for 24 hours or longer (Preparation 3). In the typical experiments, the components of the assay system were as follows in 1 ml.; 5 μmoles of s-collidine-HCl buffer (pH 7.0); 3 μmoles of D-2,3-diphosphoglyceric acid; enzyme. The mixture was incubated at 37° for 30 minutes, deproteinized with perchloric acid and the liberated inorganic phosphate was determined by Fiske-Subbarow's method (*3*).

At any step of the purification, when the enzyme preparation was dialyzed against 10^{-4} *M* ethylenediaminetetraacetic acid solution at pH 7.0, the activity was entirely lost within about 10 hours and a flocculative precipitate of protein was observed. Other chelating agents such as *o*-phenanthroline, dithizone, α, α'-dipyridyl exerted a similar effect. When the enzyme was dialyzed against Tris-HCl buffer (pH 7.0) without chelating agents, the activity was almost entirely reserved. Adding such reagents (0.001 *M*) in the assay system its phosphatase activity was entirely lost. These phenomena suggest that some metal element may be involved in the activity of glycerate 2,3-diphosphatase. The effect of mercuric ion upon the activity of different stage of purification is presented in Table I. Mercuric ion had no effect on Preparation 2, in conformation with the description of Joyce and Grisolia. However, with the use of Preparation 3 mercuric ion had the marked activating effect. Among various metal ions so far as tested (Ca^{++}, Mg^{++}, Zn^{++}, Fe^{++}, Cu^{++} and Hg^{++}), mercuric ion was the only effective cation. From the effect of chelating agents and mercuric ion, it appears likely that a trace amount of cation (presumably mercuric ion) is essential to exert enzyme activity.

The results will be published in detail elswhere.

TABLE I

The Effect of Mercuric Ion on the Phosphatase Activity

Concentration of mercuric acetate (M)	Preparation 1 s.a.=0.22	Preparation 2 s.a.=8.5	Preparation 3 s.a.=0.9
0	100	100	100
10^{-2}	28		
5×10^{-3}	5		64
10^{-3}	322	102	86
5×10^{-4}			405
10^{-4}	100	113	881,826,503
10^{-5}	100	124	108,139
10^{-6}			106,112

All activity was expressed as % activity of the control value. Specific activity (s.a.) was defined as μmoles of inorganic phosphate liberated for 1 hour per mg. protein. Protein content of each preparation in incubation mixture was 1.2, 0.12, 0.10 mg. per ml. respectively.

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